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THE PROTEINS OF GREEN LEAVES.*

I. SPINACH LEAVES.

By THOMAS B. OSBORNE and ALFRED J. WAKEMAN.

With the Cooperation of Charles S. Leavenworth and Owen L. Nolan.

(From the Laboratory of the Connecticut Agricultural Experiment Station, New Haven.)

(Received for publication, March 5, 1920.)

INTRODUCTION.

For many years it has been the practice of agricultural chemists to state the proportion of protein which the cells of the green leaves contain. These proportions have been determined, however, by conventional methods and, apparently, in no case has any corresponding quantity of protein been isolated and identified by suitable chemical methods. The reason for our present meager knowledge of the protein constituents of living plants is chiefly due to the difficulties encountered in separating the contents of the cells from their enveloping walls. Attempts to grind the fresh leaf and extract the contents of the cells with water result in mixtures that cannot be filtered clear and consequently appear to present no opportunity to obtain the protein in a state fit for chemical examination. Attempts to extract dried leaves with water yield solutions containing only a part of the total nitrogen and most of this soluble nitrogen does not belong to protein. Such solvents as are usually used for extracting proteins from animal tissues, or from seeds, fail to dissolve much of the residual nitrogen.

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, Washington, D. C.

In consequence of these facts there is at present no satisfactory evidence of the nature of the compounds containing a large proportion of the nitrogen in the green leaves of any plant. The highly specialized physiological functions of the leaf justify the expectation that it may contain constituents chemically unlike those found in other parts of the plant or in the cells of animal tissues. In how far such constituents belong to types of compounds peculiar to the leaf, or to types already well known, is scarcely known at present. It suffices merely to mention chlorophyll to call to mind one such substance. No one can foretell how many others which are likewise peculiar to the leaf may be present.

It is highly probable that the leaf is the seat of protein synthesis as already it is known to be the seat of carbohydrate synthesis. The close relations of amino-acids to carbohydrates and the known conversion of ketonic acids into amino-acids suggest chemical possibilities which deserve consideration. From this point of view the protein of the leaf may represent the original protein from which all other kinds are formed, either directly or indirectly.

Up to the present time no recent publication dealing with the protein constituents of leaves has come to our attention. In the latter part of the 18th and the early part of the 19th century the fact that the green parts of living plants contain protein was recognized by several observers and to a small extent attempts were made to study this. Later when it was found that protein could be so much more easily isolated from seeds attention was diverted from the leaf.[1] Apart from numerous attempts to apply conventional methods for distinguishing non-protein nitrogen from protein nitrogen in leaves and green plants, little appears to have been done to increase our knowledge of this subject.

Botanists have made microscopic studies of leaves and other parts of plants which furnish some facts of interest respecting their protein constituents, but at present these reveal little respecting their chemistry or value in nutrition. We have not been able to find that any one has yet isolated the protein, as

[1] For a discussion of this early history see Osborne, T. B., The vegetable proteins, Monograph on biochemistry, London, New York, Bombay, and Calcutta, 1909.

such, from leaves in a condition suitable for studying its amino-acid make-up or its nutritive value.

The experience which we have had in the past in studying the proteins of seeds, and the improved methods now available for dealing with proteins, prompted us to make this investigation which finally showed how most of the protein can be easily separated from the fresh leaf.

Although preparations thus obtained doubtless are mixtures of several individual proteins they are more suitable for a study of the nutritive value of the total protein furnished by the leaf than would be any single protein contained in this mixture. We have consequently postponed attempts to make more detailed studies of them.

The Fresh Green Leaves.

Spinach leaves were employed for the present investigation because these contain more nitrogen than do most other leaves, and also because a fresh supply can be obtained throughout the greater part of the year. The fresh leaves consist of cells filled with cytoplasm, an apparently amorphous jelly-like substance, within which can be seen the nuclei and nucleoli of the cells, the chloroplasts, and also minute particles of unknown character. In addition the vacuoli and the water-conducting cells are filled with sap, a fluid containing substances doubtless in true solution. When the walls of the cell are broken by grinding, its contents are liberated and a mixture of the nuclei, chloroplasts, cytoplasm, sap, debris of cell walls, *etc.* is obtained. The parts contributing to this mixture are no longer in the same relation to one another as when intact within the living cell; they form an amorphous mixture, in which the components no longer can be recognized under the microscope. When this mixture is diluted with one or two volumes of water and either centrifuged at high speed or passed through coarse filter paper, an opaque green, slightly viscid fluid is obtained and also a residue, consisting chiefly of the cell walls. Although the fluid part is very opaque and looks as if it contained much suspended solids, microscopic examination reveals nothing except extremely minute granules and somewhat larger, spherical particles of insoluble matter. Only occasionally

can a fragment of the disintegrated chloroplasts be seen. Apparently the fluid passing the filter contains practically nothing except the soluble and the amorphous, colloidal constituents of the cell contents. If the grinding is thorough enough the filtered fluid may contain all the contents of the cells, while the solids retained by the paper are seen to consist only of the walls of the cells and the various ducts.

On a large scale it is impossible to grind the leaves so thoroughly that all the cells are disintegrated, but it is not difficult to grind them in a Nixtamal mill so thoroughly that the filtered solution will contain by far the greater part of the cell contents.

Addition of alcohol equivalent to one-fifth of this filtered mixture produces a bulky dark green precipitate which separates, leaving a green, slightly turbid solution. After separating this precipitate with the centrifuge the further addition of alcohol to one-third of the volume of the original extract yields another large green precipitate and a solution which can be easily filtered clear. On washing these green precipitates with strong alcohol chlorophyll and other substances are easily removed, leaving an almost colorless residue. When this is treated with ether a deep yellow extract is obtained which on evaporation leaves a semi-solid fatty residue. The precipitates thus washed when air-dry are nearly colorless, friable products containing about 14 per cent of nitrogen, calculated ash-free.

A considerable part of the first precipitate is not soluble at room temperature either in an aqueous or in a 60 per cent alcoholic solution containing 0.2 to 0.3 per cent sodium hydroxide, although such solutions readily dissolve most types of proteins. If, however, the temperature of such an alcoholic solution is raised to boiling nearly all this precipitate dissolves. When the resulting solution is neutralized with acid most of the dissolved substance is precipitated and is then readily soluble at room temperature in a very slight excess of either acid or alkali. This behavior suggests that the protein was originally combined with other substances from which it is set free by hydrolysis with alkali. Proof of this, however, has not been obtained.

The second precipitate behaves like ordinary proteins, being almost completely soluble in cold dilute aqueous alkalies. Since in the original extract the protein in both the first and second

precipitates apparently exists in colloidal rather than in true solution, for convenience we shall refer to it as the "colloidal protein."

At least 40 per cent of the total spinach nitrogen belongs to this colloidal protein; about one-third to substances which are soluble in water; and a comparatively small part of the other fourth of the nitrogen belongs to chlorophyll, phosphatides, *etc.*, soluble in strong alcohol; and presumably a part of the remainder to nucleic acid.

Since this colloidal protein is so readily precipitated by a relatively small proportion of alcohol while at the same time most of the chlorophyll, phosphatides, *etc.*, which were previously insoluble in pure ether, become soluble therein, it seems possible that within the cell these and possibly other substances exist in chemical union as a complex which forms a colloidal solution extremely sensitive to the action of alcohol. If this is so the solubility and other properties of the constituents of this complex give no idea of their chemical or physical properties as they are combined within the cell. We have long been familiar with similar combination of protein with lecithin in the yolk of hen's eggs. When these are mixed with a strong solution of sodium chloride and shaken with ether until freed from all that is soluble therein an aqueous solution is obtained which can be filtered easily. The clear filtrate yields an abundant precipitate of protein when salts are removed by dialysis and this precipitate dissolves *completely* in salt solution and can be reprecipitated and redissolved without giving any indication of the presence of phosphatides or fats. If, however, it is washed with alcohol more than 20 per cent of its dry weight is dissolved and this alcoholic solution contains phosphatides and fats which in our opinion were previously chemically combined with the protein. We suspect that similar combinations exist within the cell plasma of the leaf.

The residue of the cell walls, *etc.* contained about 16 per cent of the total nitrogen of the leaves, from which it seems fair to infer that about 84 per cent of the substance of the cell contents was mechanically removed by the method of disintegration described. Evidence was obtained that most of the nitrogen in this residue of the cell walls, *etc.* belonged to constituents of unruptured cells, or of those from which the contents had not

been completely removed and was consequently protein nitrogen. As a result of this investigation it was found that about one-fourth of the solids of the spinach leaf is protein, and that one-fourth of its total nitrogen belongs to non-protein substances soluble in water.

The dry solids of the spinach leaf are much richer in protein than are any of the cereals and, in fact, some of the commercial protein concentrates such as bran, middlings, *etc.* If we remember that the cells of leaves are physiologically among the most active that we know it should not surprise us to discover that the chemical constitution of their contents is much like that of the cells of active animal tissues. The large proportion of water and cell walls makes the leaf appear to be a relatively poorer source of true nutrients than it actually is. If, however, we can learn to separate the contents of the cell from these we shall obtain a food product of very great value. This does not seem to be unattainable by means less complicated than those employed in flour milling. What may be the practical possibilities of concentrating and better utilizing the cell contents of leaves, such, for instance, as alfalfa, cannot be predicted, but the subject seems well worth considering.

EXPERIMENTAL.

1 kilo of the fresh leaves was ground by passing through a Nixtamal mill seven times. After adding 1,300 cc. of distilled water and passing through the mill three more times, most of the cells were ruptured and their contents suspended, or dissolved, in the fluid. From the results of analyzing a small aliquot of this mixture it was estimated that the ground leaves, which were treated in the way next described, contained 104.2 gm. of solids, dried at 107°, 6.0 gm. of nitrogen, and 19.3 gm. of ash. The mixture of ground leaves and water was centrifuged at high speed and the opaque, green fluid decanted from the coherent solids. These latter were washed by grinding again with 1,000 cc. of distilled water and centrifuged.

Precipitation with Alcohol.—After repeating this last treatment the three turbid solutions were each separately mixed with about one-fifth of their volume of 95 per cent alcohol. A large flocculent, dark green precipitate separated at once from the main extract, leaving a slightly turbid, green solution, but precipitates did not separate from the washings until a little sodium chloride was added. The precipitate from the second washings was small, showing that the extraction of the residue was nearly complete.

These precipitates, A, were separated by centrifuging, united, and washed with alcohol until the chlorophyll was removed. The alcoholic washings contained 4.4 gm. of solids, dried at 107°, 0.5 gm. of ash, and 0.1 gm. of nitrogen. The ash-free solids equalled 3.9 gm. Precipitate A was then washed with ether, which removed 0.8 gm. of a yellow semisolid grease, sparingly soluble in cold ether, but in boiling ether readily yielding a solution which gave an abundant precipitate when poured into acetone (lecithin ?).

Thus washed with alcohol and ether, Precipitate A was dried in the air at room temperature. It contained 0.5 gm. of ash, 1.1 gm. of nitrogen, and 0.034 gm. of phosphorus; its weight, ash- and moisture-free, was 7.9 gm.

More alcohol was added to the filtrates from Precipitate A produced by one-fifth volume of alcohol, until the volume was equal to one-third that of the original extract. This caused a second flocculent green precipitate, B, in each solution. These precipitates were united and washed with alcohol until the chlorophyll was removed. The alcoholic washings contained 2.2 gm. of solids, dried at 107°, 0.1 gm. of ash, and 0.04 gm. of nitrogen. The ash-free solids equalled 2.1 gm.

TABLE I.

	Precipitate A.	Precipitate B.
	per cent	*per cent*
Ash	7.4	0.2
Nitrogen	12.9 = 13.7 ash-free.	14.9 = 14.9 ash-free.
Phosphorus	0.4 = 0.4 "	0.3 = 0.3 "

Precipitate B was then washed with ether which extracted 0.5 gm. of a deep yellow grease. This appeared to contain more of the yellow oil and less of the colorless lecithin than did the ether extract of Precipitate A. Thus washed and dried in the air at room temperature, Precipitate B contained 0.03 gm. of ash, 1.6 gm. of nitrogen, and 0.022 gm. of phosphorus. The ash-free substance, dried at 107°, equalled 10.4 gm.

Since Precipitates A and B formed so much of the total solids of the leaf and appeared to consist mostly of protein they deserve special examination. The figures given in Table I facilitate a comparison of the data just given.

Probably Precipitate A contained more ash and less nitrogen than Precipitate B, because it carried down fine particles suspended in the turbid extract.

When hydrolyzed by boiling with 20 per cent hydrochloric acid for 14 hours these precipitates showed proportions of nitrogen in different forms as given in Table II.

The distribution of nitrogen among the several types is similar to that of most proteins with the exception of the humin nitrogen which is so very much greater as to suggest the presence of carbohydrate.

Examination of the Precipitates.—Although these figures indicate that Precipitates A and B consisted largely of protein they afford no satisfactory basis for an estimate of its actual proportion. Accordingly 3.48 gm. of Precipitate A and 4.67 gm. of Precipitate B were dissolved as far as possible by agitating with about 200 cc. of 0.2 per cent sodium hydroxide solution and removing the undissolved part by centrifuging. After repeating this extraction five successive times the clear alkaline solutions were united and neutralized with dilute hydrochloric acid. The precipitate which separated was washed with dilute alcohol, then with ether, and dried at 107°.

In the united filtrate and washings organic matter and nitrogen were determined. After removing alcohol, protein also was determined in this solution by saturating with ammonium sulfate. The difference between the total nitrogen and the ammonia nitrogen in the solution of the precipitate produced by the sulfate showed that 0.17 gm. of protein (N × 6.25) which had not been precipitated by neutralizing was present in this filtrate. The total organic solids in this filtrate weighed 1.5390 gm., hence at least

TABLE II.

	Precipitate A.	Precipitate B.
	per cent	*per cent*
Nitrogen as ammonia	7.9	7.2
Basic nitrogen	22.5	24.2
Humin "	7.5	5.0
Other " (by difference)	62.1	63.6
Total	100.0	100.0

1.37 gm. or 16.8 per cent of non-protein organic matter must have been present in Precipitates A and B before treatment with the aqueous alkali. This filtrate contained 0.08 gm. of nitrogen of which 0.03 gm. was precipitated by saturating with ammonium sulfate from which it appears that non-protein nitrogenous matters also were present. A portion of this filtrate was hydrolyzed by boiling with 1 per cent hydrochloric acid for 2 hours and sugar estimated by reduction of cupric oxide. It was thus determined that the entire filtrate contained carbohydrate equal to 0.09 gm. of dextrose, or 0.10 gm. of xylose, or 0.08 gm. of xylan, from which it would appear that no considerable quantity of carbohydrate was present.

The residues which failed to dissolve in the aqueous alkaline solutions were extracted three successive times by boiling for a few minutes with 60 per cent alcohol containing 0.3 per cent sodium hydroxide. The last extract yielded no precipitate when neutralized with acid. The two first clear extracts were neutralized with hydrochloric acid, and the precipitates washed first with dilute, then with strong alcohol, and finally with ether, and dried at 107°. Nitrogen and organic matter were determined in the

precipitates and also in the filtrates and washings. The residues which failed to dissolve in the hot alkaline alcohol were washed with alcohol and ether and dried at 107°. Table III gives the results obtained.

These figures show that while 96 per cent of Precipitate B was soluble in aqueous alkali only 56 per cent of Precipitate A dissolved therein. Most of the insoluble part, however, did dissolve when boiled with the alkaline alcohol. Not all the nitrogen dissolved by the alkaline solutions was precipitated by neutralizing, for the filtrates contained nitrogen, a part of which was protein nitrogen precipitable by saturating with ammonium sulfate as has just been shown. While Precipitates A and B unquestionably contained some non-protein substance, 72 per cent of the ash-free substance of Precipitates A and B combined was recovered in the neutralization precipitates.

TABLE III.

			Precipitate A.	Precipitate B.
			gm.	gm.
Aqueous alkaline extract	Precipitate	Organic matter	4.571	
		Nitrogen	0.697	
	Filtrate	Organic matter	1.539	
		Nitrogen	0.078	
Alcoholic " "	Precipitate	Organic matter	0.950	0.095
		Nitrogen	0.145	0.014
	Filtrate	Organic matter	0.267	0.048
		Nitrogen	0.028	?
Insoluble residue		Organic matter	0.293	0.053
		Nitrogen	0.012	0.002
		Ash	0.091	?

The precipitate produced by neutralizing the united aqueous alkaline solutions of Precipitates A and B contained 1.47 per cent of ash, 15.25 per cent of nitrogen, and 0.09 per cent of phosphorus, calculated for the dry, ash-free substance. When hydrolyzed by boiling with 20 per cent hydrochloric acid for 14 hours proportions of the different forms of nitrogen were found as given in Table IV.

These figures agree quite closely with those found for Precipitate B and show that after solution in alkali and reprecipitation with acid the relatively low nitrogen content of this protein remains the same. This fact, together with the large amount of humin nitrogen, suggests that the protein may either be combined with, or contaminated by, carbohydrate or else with some group containing carbohydrate. A determination of pen-

tose by distillation with hydrochloric acid gave a phloroglucin precipitate equal to 2.7 per cent of the protein as pentose or to 2.4 per cent as pentosan.

The uniform percentage of nitrogen found in the neutralization precipitates from the aqueous alkali, the alcoholic alkali, and, as will be shown later, from alkaline alcoholic extracts of another lot of dried spinach leaves (see p. 19), namely 15.25, 15.27, 15.52, and 15.41 respectively, indicates that some non-protein substance may be combined rather than admixed with the protein. A critical examination, however, of larger quantities of this protein will be needed to settle this question.

The Water-Soluble Constituents of the Leaves.—The united filtrates from the precipitate produced by one-third volume of alcohol measured 7,115 cc., a volume so large, in comparison with the amount of water-soluble constituents of the leaves, that there is little probability that any substances, actually soluble in water, were precipitated by the alcohol.

This solution was concentrated *in vacuo*, below 65°, to 2,650 cc. During concentration a very little substance separated which was not removed.

TABLE IV.

	Total N.	Protein.
	per cent	*per cent*
Nitrogen as ammonia	7.50	1.32
Basic nitrogen	22.49	3.96
Humin "	5.97	1.05
Other " (by difference)	64.04	8.92
Total	100.00	15.25

Determinations made on aliquots of 500 cc. showed that the entire solution contained 2.07 gm. of nitrogen, and, after deducting the sodium chloride which had been added, 45.57 gm. of solids containing 15.69 gm. of ash. The ash-free solids, therefore, were equal to 29.88 gm., and the total solids to 43.7 per cent of the leaves dried at 107°.

The remainder of this solution was saturated with ammonium sulfate, the precipitate treated with water, and the insoluble part removed by centrifuging, washed thoroughly with water, alcohol, and ether, and dried at 107°.

In terms of the entire water extract this insoluble fraction weighed 1.76 gm., and contained 0.227 gm. of nitrogen and 0.102 gm. of ash = 5.8 per cent of the fraction. The ash-free substance, which was almost completely soluble in dilute alkali and gave a good biuret reaction, weighed 1.66 gm. and contained nitrogen equal to 1.42 gm. of protein ($N \times 6.25$) or to 85 per cent of the organic matter. Presumably this was mostly protein rendered insoluble in water by the treatment to which it had previously been subjected.

The solution of the ammonium sulfate precipitate was again saturated with this salt, and the precipitate freed as far as possible from sulfate by pressing between filter papers, and then dissolved in water. The difference between the total nitrogen and the ammonia nitrogen found in the solution, representing nitrogen of proteoses, was 0.286 gm., calculated for the whole water extract, or 1.79 gm. of proteose (N × 6.25).

The Insoluble Residue of the Leaves.—The microscope showed that the residue of the spinach leaves, which had been extracted three times with water, as already described, consisted chiefly of broken walls of cells and ducts. Among these were a few unruptured cells and some broken cells still retaining a part of their original contents. It was evident that the latter had been largely, though by no means completely, removed. This residue was then washed with alcohol and ether until chlorophyll was extracted. These washings contained 0.083 gm. of nitrogen, 3.212 gm. of solids, and 0.40 gm. of ash. The ash-free solids equalled 2.812 gm.

The washed residue, when dried at room temperature in the air, formed an almost perfectly white powder, equal to 32.25 gm., dried at 107°, and contained 0.960 gm. of nitrogen and 4.15 gm. of ash.

A quantity of this cell residue, equal to 9.243 gm., dried at 107°, was boiled with 500 cc. of 1 per cent hydrochloric acid for 4 hours and, after centrifuging, the residue was treated twice more in the same way. Pentosans and insoluble proteins were thus converted into soluble products by this mild hydrolysis. The solution contained 5.05 gm. of organic solids, 0.234 gm. of nitrogen, equal to 17.79 gm. and 0.81 gm. respectively in the entire residue. Hydrolysis had, therefore, rendered soluble 55.1 per cent of the solids and 85.0 per cent of the nitrogen. The part still undissolved, after washing with water and drying at 107°, weighed 2.636 gm. equal to 9.2 gm., calculated for the entire cell residue, or to 8.8 per cent of the spinach leaves. This residue contained 0.7 gm. of ash and 0.11 gm. of nitrogen equal to 0.67 gm. of protein, calculated for the original cell residue. Deducting these, the ash- and protein-free residue was equal to 7.5 per cent of the spinach leaves. This is decidedly more than the 5.5 per cent of crude fiber found in another sample by the conventional method of the agricultural chemists. The part undissolved by the boiling dilute acid also contained 0.031 gm. of nitrogen, equal to 0.108 gm. in the entire cell residue, or to 1.8 per cent of the nitrogen of the fresh spinach leaves.

The first acid extract, which contained 94 per cent of the total soluble solids and 92 per cent of the soluble nitrogen, was concentrated *in vacuo* to one-tenth of its volume. A considerable precipitate separated which partly dissolved on washing with water. The undissolved part, dried at 107°, weighed 0.747 gm. and consisted largely of inorganic matter. The soluble part reduced cupric oxide equivalent to 7.4 gm. of xylose calculated for the entire residue. If the second and third acid extracts contained the same proportion of xylose as did the first extract, the total xylose would be 7.5 gm., equal to 6.5 gm. of xylan.

Of the total nitrogen 19.4 per cent was free amino nitrogen and 4.9 per cent ammonia nitrogen. After boiling with 20 per cent hydrochloric acid

for 10 hours the amino nitrogen was increased to 56.1 per cent of the total and the ammonia nitrogen to 7.4 per cent. These proportions are like those yielded by the colloidal protein and indicate that most of the nitrogen extracted from the cell residue by boiling with dilute acid was derived from this protein. Assuming this to be the case the total protein thus extracted would be equal to 5.10 gm. calculated for the entire cell residue. The protein and xylan thus estimated together are equal to 11.65 gm., leaving 6.14 gm. of organic substances of undetermined nature, or 5.9 per cent of the spinach leaves.

Table V summarizes the results of the preceding analysis of the fresh spinach leaves.

TABLE V.

	Solids ash-free.	Nitrogen.
	gm.	*gm.*
Soluble in alcohol and ether:		
Chlorophyll, lecithin, fat, *etc.*	10.1	0.24
Soluble in water:		
Proteose (N × 6.25)	1.8	0.29
Coagulable protein	1.4	0.23
Non-protein organic substances	26.7	1.56
In colloidal suspension in water	18.3	2.62
Extracted by boiling 1 per cent HCl	17.8	0.81
Residue of cell walls, *etc.*	9.2	0.11
	85.3	
Inorganic substances	19.3	
Total	104.6	5.86
Taken for analysis	104.2	6.02

We have shown that the precipitates produced by alcohol in the original turbid extracts consist chiefly of protein and, judging from their phosphorus, contain very little nucleic acid. We shall, therefore, not be far from the truth in estimating this protein by multiplying the nitrogen of these precipitates by 6.25. We have also shown that substances reducing copper oxide equal to 6.54 gm. of xylan and nitrogen, probably mostly of protein origin, equal to 5.1 gm. of protein are extracted from the residue of cell walls by boiling 1 per cent hydrochloric acid. The sum of the pentosan and protein, thus calculated, subtracted from that of the total organic solids of the acid extract gives 6.14 per cent of organic substances of still unknown character. The nature of the small amount of the nitrogenous substance remaining in the residue after extracting with boiling 1 per cent hydrochloric acid was not determined, but we have assumed

that this was protein. Table VI gives the calculation of the results of this analysis in percentage of ash-free substances in the fresh spinach leaves.

These figures show that about one-fourth of the solids, as well as of the nitrogen, of the fresh spinach leaf belongs to non-protein, water-soluble substances, the nature of which has not yet been determined. Only a very small part of these contains the R-$CONH_2$ group because the ammonia, obtained by boiling the dried leaves with 60 per cent alcohol containing 0.3 per cent sodium hydroxide, amounted to only 1.6 per cent of the total nitrogen. About 17 per cent of the nitrogen in the water-soluble, non-protein substances is free amino nitrogen.

TABLE VI.

	Solids ash-free.	Nitrogen.
	per cent	*per cent*
Soluble in alcohol and ether:		
Chlorophyll, lecithin, fat, *etc.*	9.7	4.0
Soluble in water:		
Proteose (N × 6.25)	1.7	4.8
Coagulable protein (N × 6.25)	1.4	3.8
Non-protein organic substances	25.6	25.9
In colloidal suspension in water:		
Protein (N × 6.25)	15.7	43.5
Non-protein substance (pentosans ?)	1.8	
Extracted by boiling 1 per cent HCl:		
Protein (N × 6.25)	4.9	13.6
Pentosans (xylan)	6.3	
Other organic substances	5.9	
Insoluble residue:		
Protein (N × 6.25)	0.6	1.7
Cellulose, *etc.*	7.5	
	81.1	
Inorganic substances	18.5	
Total	99.6	97.3

How much of the remaining three-fourths of the nitrogen belongs to protein is not precisely established by the data here presented, but it is certain that the proportion of non-protein nitrogen is small. Most of the nitrogen of the Precipitates A and B, produced by alcohol as already shown, unquestionably belongs to protein. The small amount of phosphorus found in them limits the proportion of nucleic acid to not more than 3 or 4 per cent of these precipitates.

We have already given reasons for believing that most of the nitrogen in the cell residue belongs to protein. In so far as this nitrogen is derived from broken cells it is likely that most of the water-soluble protein escaped

and passed into solution; and in so far as it is derived from unbroken cells very little can be assigned to water-soluble proteins because these contain so small a proportion of the total nitrogen of the leaf. Consequently we shall not be far from right in assuming that all this nitrogen belongs to the colloidal protein which had escaped extraction through failure to break up *all* the cells by grinding. The proportion of the different proteins in the fresh spinach leaves can be stated to be approximately as follows:

	Dry leaf. *per cent*
Proteose	1.7
Coagulable protein	1.4
Colloidal "	21.3
Total protein	24.4

From these figures it appears that the spinach leaf must be regarded as a food rich in protein.

The Dried Leaves.

Since many green foods, such as alfalfa or clover, consist chiefly of leaves and when dried and cured are fed as hay it seemed desirable therefore to examine the spinach leaves after they had been dried in a current of warm air below 60° and to compare the results obtained with those yielded by the green fresh leaves. Unfortunately, no supply of the lot of leaves used in the fresh state was available and it was necessary to use leaves which had been gathered early in the summer and were much less mature. As we intend later to apply the methods developed in this preliminary investigation to leaves of greater agricultural importance we have decided to publish the results which we obtained, because they show that drying at a low temperature has far less effect than one might expect on the nature or solubility of the constituents of the leaves.

The proportion of substances soluble in water is a little greater in the dried leaf than in the green, both protein and non-protein substances contributing to this small excess. The distinctly greater amount of proteose found in the extract of the dried leaf compared with the fresh indicates a slight autolysis during drying but proof that such a change actually occurs can only be secured by comparing the results obtained with parts of the same sample.

The percentage of the non-protein organic substances extracted from the dried leaves with water is the same as that remaining in solution after precipitating the turbid extract of the green leaves by the addition of one-third volume of alcohol. In both cases the percentage of the total nitrogen soluble in water is also alike. The amount of the colloidal protein obtained from these two sources was likewise nearly the same.

The results of this comparison justify the conclusion that data obtained with leaves carefully dried at a low temperature closely represent the composition of the leaf in its fresh, green condition.

EXPERIMENTAL.

The leaves, with about 1½ inches of stems attached, were carefully washed and dried in a current of warm air at a temperature not exceeding 60°. They were then ground to a very fine powder and successively extracted with ether, alcohol, and boiling water as follows:

Ether.—Two lots, I and II, of the air-dry spinach leaves were used. Each contained 18.22 gm. of solids, 0.866 gm. of nitrogen, and 4.44 gm. of ash, calculated for the leaves dried at 107°. Lot I was dried in the extraction capsule for 14 hours at 100°. It was covered with a plug of absorbent cotton, likewise dried, and extracted in the Soxhlet apparatus from which all moisture was removed by drying in the oven. The spinach leaves were then continuously extracted for 18 hours with ether which had been distilled over sodium. For comparison, Lot II in the air-dry condition was extracted with ordinary U.S.P. ether for the same time.

Alcohol.—Lot I was next extracted with absolute alcohol, Lot II with ordinary commercial alcohol, containing 6.5 per cent of water.

Boiling Water.—After thus removing everything soluble in ether, as well as in alcohol, Lots I and II were each extracted with about 400 cc. of boiling water five successive times, the residues being separated by centrifuging at high speed. The united extracts were concentrated to 500 cc. and nitrogen, solids, and ash determined in aliquots. The results of these extractions are given in Table VII.

The solids thus extracted by these several solvents were equal to percentages of the dried spinach leaves as shown in Table VIII.

The nitrogen extracted was equal to percentages of the total nitrogen of the dried spinach leaves as given in Table IX.

The spinach leaves contained 22.51 per cent of ash. The percentages of the total mineral matters extracted are given in Table X.

These figures show that these solvents extracted about one-half of the solids, one-fourth of the nitrogen, and nine-tenths of the mineral constituents.

Pure Ether.—Pure ether extracts nitrogen equivalent to 0.33 per cent of chlorophyll (N × 16.2) in the dry spinach leaves, or about 10 per cent

TABLE VII

	I. Pure ether.		II. U.S.P. ether.	
	gm.	*per cent*	*gm.*	*per cent*
Solids	0.5389	2.96	0.8545	4.70
Nitrogen	0.0036	0.02	0.0100	0.06
Ash	0.0093	0.06	0.0162	0.09
	I. Absolute alcohol.		II. 93.5 per cent alcohol.	
Solids	0.6255	3.43	1.0216	5.59
Nitrogen	0.0168	0.09	0.0486	0.21
Ash	0.0843	0.46	0.1851	1.01
	I. Boiling water.		II. Boiling water.	
Solids	8.1500	44.73	7.5940	41.51
Nitrogen	0.2190	1.20	0.1860	1.02
Ash	3.9375	21.61	3.7970	20.84

TABLE VIII.

Percentage of Solids Extracted from Dried Spinach Leaves by Various Successive Solvents.

Lot I.		Lot II.	
	per cent		*per cent*
Ether, pure	2.96	Ether, U.S.P.	4.70
100 per cent alcohol	3.43	93.5 per cent alcohol	5.59
Boiling water	44.73	Boiling water	41.51
Total	51.12		51.80

TABLE IX.

Percentage of the Total Nitrogen Extracted from Dried Spinach Leaves by Various Successive Solvents.

Lot I.		Lot II.	
	per cent		*per cent*
Ether, pure	0.40	Ether, U.S.P.	1.15
100 per cent alcohol	1.94	93.5 per cent alcohol	5.61
Water	25.29	Water	21.48
Total	27.63		28.24

of the solids soluble in pure, dry ether. Commercial U.S.P. ether extracted about three times as much nitrogen from the air-dry leaves as did dry ether. In terms of chlorophyll this would equal about 18 per cent of the solids extracted or 0.85 per cent of the leaves. Assuming that all this nitrogen belongs to chlorophyll, 2.63 and 3.85 per cent respectively would belong to other substances extracted by the two kinds of ether. It is evident that the alcohol and water in the U.S.P. ether has a marked effect on the solubility of these lipoids and but little on that of the inorganic matters.

Absolute Alcohol.—Absolute alcohol, after pure ether, extracted only 2.3 per cent of the spinach nitrogen. If this were all chlorophyll nitrogen about 1.5 per cent of the leaves, or about 43 per cent of the total solids of the alcoholic extract, might be chlorophyll. Since the solids extracted by absolute alcohol contained 13.5 per cent of mineral matter it is possible that, besides chlorophyll, salts or inorganic acids are also removed. The solids extracted by absolute alcohol dissolved readily in ether, although

TABLE X.

Percentage of the Total Mineral Ash Extracted from Dried Spinach Leaves by Various Successive Solvents.

Lot I.		Lot II.	
	per cent		*per cent*
Ether, pure	0.21	Ether, U.S.P.	0.36
100 per cent alcohol	1.90	93.5 per cent alcohol	4.17
Water	88.70	Water	85.52
Total	90.81		90.05

ether-soluble substances had previously been very completely removed. When this ether solution was poured into an excess of acetone a coherent deposit formed on standing, which, when treated with ether, was not completely soluble. On centrifugation a greasy sediment formed which, for the most part, was soluble in absolute alcohol.

Apparently the excess of nitrogen removed by commercial alcohol belongs to substances soluble in boiling water, because the leaves previously extracted with pure ether and then with absolute alcohol, when exhausted with boiling water, yielded extracts containing a correspondingly greater amount of nitrogen. The excess of nitrogen extracted by commercial ether over that extracted by pure ether possibly belongs mostly to chlorophyll set free by the alcohol which the commercial ether contains.

Cold Water.—The residue remaining after extracting with ether, alcohol, and boiling water contained nearly three-fourths of the nitrogen of the leaf. Since this residue had been boiled with water it was not suitable for further examination of its proteins. Accordingly, after extracting dry spinach leaves with absolute ether and then with absolute alcohol, 20 gm.

of the dry residue (equal to 20.2 gm. of the original dry leaves and containing 0.95 gm. of nitrogen) were digested with 400 cc. of water at room temperature and then ground in a Nixtamal mill. After separating the solids by centrifuging, the residue was treated six more times in the same way. The last extract contained 0.2960 gm. of solids, dried at 107°, from which it is evident that it is practically impossible to remove completely all that is soluble in water.

The flocculent, insoluble matter that separated from the united extracts during concentration, when dried at 107°, weighed 0.6193 gm., and contained 0.0564 gm. of nitrogen and 0.0992 gm. of ash. This product contained 16.02 per cent of ash and only 10.85 per cent of nitrogen, calculated for the ash-free substance, equal to 5.94 per cent of the spinach nitrogen and to 0.3525 gm. of protein (N × 6.25) corresponding to 1.74 per cent of the dried spinach leaves.

The filtrate contained 8.984 gm. of solids, dried at 107°, equal to 44.47 per cent of the original dried leaves. The total solids thus extracted were equal to 47.53 per cent of the leaves. These contained 4.1820 gm. of ash and 0.308 gm. of nitrogen, equal to 32.42 per cent of the spinach nitrogen. Thus 38.36 per cent of the spinach nitrogen was soluble in water. The remainder of this filtrate was saturated with ammonium sulfate, the precipitate redissolved in water, and its solution again saturated with this salt. The precipitate was pressed on filter paper, dissolved in water, and ammonia nitrogen and total nitrogen were determined in the solution. By difference, 0.1075 gm. of nitrogen representing the nitrogen of proteoses was thus found. This corresponds to 11.32 per cent of the total nitrogen, or to proteose (N × 6.25), equal to 3.33 per cent of the original dried leaves. Deducting the nitrogen of the proteose from the total nitrogen of the extract leaves 21.1 per cent of water-soluble non-protein nitrogen in the spinach leaves.

Alkaline Solutions.—The residue of the leaves after extraction with cold water, when heated with either hot or cold dilute sodium hydroxide solution, yielded only a small part of its nitrogen to the extract. Cold 1 per cent hydrochloric acid also proved ineffectual. It was found, however, that by boiling for a short time with 60 per cent alcohol containing 0.3 per cent sodium hydroxide a relatively large amount of protein was extracted.

Accordingly spinach leaves, which had been dried in a current of warm air at 60°, were extracted with absolute ether and then with a mixture of ether and alcohol until freed from all that was soluble therein. These removed 7.8 per cent of solids. A quantity of the air-dry residue, thus extracted, equal to 9.951 gm. of the original leaves and containing 0.51 gm. of nitrogen was heated to boiling for about 5 minutes with 500 cc. of 60 per cent (by weight) alcohol containing 0.3 per cent of sodium hydroxide. The residue was washed twice by boiling with 400 to 500 cc. of 60 per cent alcohol. The extract and washings were made faintly acid to litmus with hydrochloric acid, concentrated *in vacuo* to about 400 cc., and then made clear by the cautious addition of very dilute sodium hydroxide solution.

Hydrochloric acid was then added until the precipitate separated as completely as possible. This was washed with 60 per cent alcohol, then with strong alcohol and ether, and dried at 107°. It weighed 1.78 gm. and contained 1.12 per cent of ash. The ash- and moisture-free substance weighed 1.77 gm., equal to 17.7 per cent of the original spinach leaves, and contained 0.274 gm. of nitrogen equal to 15.5 per cent of the precipitate or to 53.7 per cent of the total spinach nitrogen.

The filtrate contained 0.135 gm. of nitrogen, equivalent to 26.4 per cent of the spinach nitrogen and 0.008 gm. of nitrogen as ammonia, equal to only 1.5 per cent of the total nitrogen of the spinach leaves. The proportion of nitrogen in this filtrate was, therefore, approximately the same as that extracted by boiling water. The residue, after extraction with alkaline alcohol, weighed 4.64 gm., equal to 46.6 per cent of the original spinach leaves. It contained 0.077 gm. of nitrogen, equal to 15.1 per cent of the spinach nitrogen.

Since so large a proportion of the nitrogen remained in this residue another extraction was made using 60 per cent alcohol containing 0.4 per cent sodium hydroxide. The ash- and moisture-free protein precipitate obtained weighed 1.77 gm., equal to 17.8 per cent of the original dry spinach. This contained 0.274 gm. of nitrogen, equal to 15.4 per cent of the ash- and moisture-free protein or to 53.8 per cent of the spinach nitrogen. The filtrate from this precipitate contained 0.146 gm. of nitrogen equal to 28.5 per cent of the spinach nitrogen. The ammonia nitrogen was 0.008 gm., equal to 1.6 per cent of the spinach nitrogen.

Since an appreciable part of the ammonia might have been lost from the alkaline extract before this had been acidified we boiled the dried spinach leaves with 60 per cent alcohol containing 0.3 per cent sodium hydroxide and determined the total ammonia liberated, under conditions which precluded losses, to be the same as previously found. Since it is possible that a part of this ammonia originated from the non-protein, water-soluble nitrogenous substances which constitute about one-fourth of the total nitrogen of the spinach leaf it is probable that the protein lost little, if any, of its amide nitrogen during the short time it was heated with the alkaline alcohol. This conclusion is further supported by the fact that the nitrogen content of the protein thus extracted from the dry leaf was the same as that of the colloidal protein obtained from the fresh green leaf by means of cold dilute aqueous alkali.

The residue which had been extracted with alkaline alcohol, when dried at 107°, weighed 4.534 gm., equal to 45.5 per cent of the original leaves, and contained 0.068 gm. of nitrogen, equal to 13.3 per cent of the total nitrogen of the spinach leaves. The close agreement between the results of these extractions is shown by the figures in Table XI.

As these preparations contained the same amount of nitrogen as did the colloidal protein from the fresh leaves, and in other respects were so similar, it seems fair to presume that they are one and the same protein, or the same mixture of proteins. The conditions under which these prepar-

ations were extracted make it improbable that they contained any of the proteose and should they contain any of the coagulable protein the amount of this present in the spinach leaf is too small to contribute appreciably to the proportion of the nitrogen recovered in them. We, therefore, feel justified in concluding that at least one-half of the spinach nitrogen is contained in the colloidal protein. How much more of this escaped extraction it is impossible to say. The residue after extraction with alkaline alcohol contained 15 per cent of the spinach nitrogen, but, in view of the very thorough extraction, it is improbable that much of this was protein nitrogen, unless this was still retained within unruptured cells.

TABLE XI.

	60 per cent alcohol.			
	Plus 0.3 per cent NaOH.		Plus 0.4 per cent NaOH.	
	Per cent of solids.	Per cent of nitrogen.	Per cent of solids.	Per cent of nitrogen.
Protein	17.9	53.7	18.0	53.8
Filtrate	27.7	26.4	28.7	28.5
Residue	46.6	15.1	45.5	13.3
Ether solution	7.8	2.3	7.8	2.3
Total	100.0	97.5	100.0	97.9

DISCUSSION.

Comparison of the Results of Extracting the Fresh and Dried Spinach Leaves.

The proportion of solids as well as of nitrogen extracted from the dried leaves by ether, U.S.P., and 93 per cent alcohol and then by cold water is the same as that extracted from the fresh green leaves by the very different method described in the earlier part of this paper. This is shown in Table XII.

The water extract of the fresh leaves contained a little larger proportion of organic and a smaller proportion of inorganic solids than did that of the dry leaves (Table XIII).

The fresh leaves contained a much smaller percentage of mineral constituents than did the dried leaves which undoubtedly accounts for the difference in the inorganic substances shown by the figures in Table XIII.

The proportion of organic solids from the two sources is nearly the same. If, however, the ash-free solids of the ether-alcohol extracts are added the difference becomes not inconsiderable, namely 6.7 per cent, the total from the fresh leaves being 38.39 per cent and from the dry leaves 31.69 per cent. This excess of substance soluble in alcohol contained substances also soluble in water as we found that the residue left by evaporating off the alcohol nearly all dissolved in ether and that this solution left a residue largely soluble in water. No clue, however, has been obtained as to the nature of this water-soluble substance.

TABLE XII.

	Solids.		Nitrogen.	
	Fresh leaves.	Dry leaves.	Fresh leaves.	Dry leaves.
	per cent	*per cent*	*per cent*	*per cent*
Soluble in ether and alcohol	10.66	6.39	3.98	0.28
" " water, total solids	43.76	47.54	34.40	38.36
Total extracted	54.42	53.93	38.38	38.64

TABLE XIII.

	Fresh leaves.	Dry leaves.
	per cent	*per cent*
Organic solids soluble in water	28.67	26.36
Inorganic " " " "	15.06	21.18
Total soluble solids	43.73	47.54

During the early stages of the drying process, when the leaves are exposed to a temperature favorable for autolysis, changes of considerable magnitude may occur. Table XIV shows, however, that the proportion of the coagulable protein was only slightly greater and that of the proteose not very much greater in the extracts from the dry leaf than in those from the fresh, while the proportion of non-protein nitrogen was practically the same.

In how far these minor differences were due to autolytic changes occurring during drying or to differences in the composition of the two lots of leaves cannot be determined until new experiments are made.

We have shown that dilute alcohol precipitates 43.5 per cent of the spinach nitrogen from the colloidal solution obtained by grinding the fresh leaves with water, and that most of this is protein nitrogen. The residue of cell walls, *etc.* still contained nitrogen equal to 15.2 per cent of the total nitrogen of the leaf which for the most part is almost certainly protein nitrogen. As there is good reason to believe that most of this belongs to proteins of the same character as those found in the extract (see p. 12) we shall not go far wrong in assuming that in the fresh leaves at least 58 per cent of the spinach nitrogen, or 21.2 per cent of the spinach solids, belongs to this protein.

The protein extracted from the dried leaves by alkaline alcohol is unquestionably the same as that precipitated from the aqueous extracts of the fresh leaves by the addition of alcohol, and which

TABLE XIV.

	Solids.		Nitrogen.	
	Fresh leaves.	Dry leaves.	Fresh leaves.	Dry leaves.
	per cent	*per cent*	*per cent*	*per cent*
Proteose (N × 6.25)	1.7	3.3	4.8	11.3
Coagulable protein (N × 6.25)	1.4	1.7	3.8	5.9
Non-protein organic matter	28.7	26.4	25.9	26.4
Total	31.8	31.4	34.5	43.6

we have provisionally designated as the colloidal protein. This protein from the dried leaves contained a little smaller proportion of the total spinach nitrogen than did the colloidal protein obtained from the water extract of the fresh leaves, but this deficit was made up by a correspondingly larger proportion of water-soluble proteins, possibly formed by autolysis during drying.

In estimating the approximate proportion of the colloidal protein in either the fresh or dry leaves we have not taken into account its possible contamination with insoluble non-protein nitrogenous compounds. Doubtless the nuclei of the cells contain nucleic acid, but either the amount of this is so small, or else its properties are so different from the nucleic acids with which we have been familiar, as to yield negative results with such tests as were employed. With the above reservations and assuming that these

undetected non-protein nitrogenous substances contain too small a proportion of the spinach nitrogen to have importance for this preliminary investigation we can state the proportion of the spinach nitrogen among the various groups as given in Table XV.

TABLE XV

	Nitrogen	
	Fresh leaves.	Dry leaves.
	per cent	*per cent*
Soluble in ether and alcohol	2.3	2.3
" " water:		
Non-protein substances	25.9	26.4
Proteose	4.8	11.3
Coagulable protein	3.8	5.9
Insoluble in water:		
Colloidal protein	58.7	53.8
Total	95.5	99.7

Stated in terms of percentage of the dry solids of the spinach leaf Table XVI shows the proportions of the different proteins ($N \times 6.25$) obtained from the fresh and dry leaves respectively.

TABLE XVI.

	Fresh leaves.	Dry leaves.
	per cent	*per cent*
Proteose	1.7	3.3
Coagulable protein	1.4	1.7
Colloidal "	21.2	22.8
Total protein	24.3	27.8

It thus appears that protein substances contain approximately 67 per cent of the total nitrogen of the fresh leaves and 71 per cent of that of the dried leaves, or practically two-thirds of the nitrogen in each case. While these figures must be accepted as only roughly approximate we believe they afford a better basis for assuming that the greater part of the nitrogen of the leaf actually belongs to protein substances than has heretofore been presented. The conventional methods in common use have fur-

nished support for such an assumption, but such indirect evidence as they afford has little value unless supported by chemical identification.

Concentration of the Spinach Protein.

By applying the results of this investigation to spinach leaves we have made a protein concentrate which we are now feeding to albino rats with marked success. This product was prepared by extracting the dried and finely ground leaves with boiling water whereby the large proportion of soluble salts and organic substances was removed. The loss of protein incurred by thus extracting was small since proteoses form but about 10 per cent of the proteins of this leaf. The dry residue, almost exactly one-half of the solids of the leaf, contained nitrogen equal to 39 per cent of protein (N × 6.25). We thus have as rich a protein concentrate as cottonseed meal.

If, as seems probable, similar products can be made from other green leaves it ought to be possible to feed these as the sole source of nitrogen and thereby increase our knowledge of the nutritive value of a class of proteins about which at present we know almost nothing. Possibly we shall also be able to learn something of the nutritive value of the water-soluble constituents. A new field for investigation appears to be thus opening which promises to be fruitful.

SUMMARY.

When fresh green spinach leaves are ground with water the contents of their cells are set free. The cell walls and other suspended matters can then be removed by filtering through soft paper or by centrifuging at high speed. A green turbid extract is thus obtained which contains the contents of the cells. The microscope shows that chloroplasts and all other formed elements have disappeared, only the most minute particles being visible, suspended in this fluid.

The addition of about one-third volume of alcohol to this extract causes a voluminous green precipitate to separate in large flocks leaving a clear solution.

The filtrate from this precipitate contains the water-soluble constituents of the cells, forming nearly one-half of the solids of the leaf. Only a small part of these is protein, proteoses being equal to only 1.7 per cent of the total solids of the leaf and proteins coagulable by heat to only 1.4 per cent. The non-protein organic substances are equal to about 28 per cent of the solids of the leaf and contain about one-fourth of its total nitrogen. The balance of these water-soluble constituents consists of inorganic matters.

The green precipitates produced by alcohol contain chlorophyll phosphatides, and fats which can be extracted by alcohol and ether, leaving a residue equal to about 20 per cent of the solids of the leaves and containing nearly 50 per cent of their nitrogen. This part of the alcohol precipitate consists almost wholly of protein, but, unlike other native proteins, it is not readily soluble in aqueous alkaline solutions at room temperature. When boiled for a few minutes with 60 per cent alcohol containing 0.3 per cent sodium hydroxide it dissolves and when this solution is neutralized with acid nearly all the protein is precipitated and is then readily soluble in a slight excess of either acid or alkali. This latter precipitate, which we have provisionally designated the colloidal protein, has the properties of pure protein, and contains 15.25 per cent of nitrogen. As it yields furfurol equivalent to 2.5 per cent of pentose this protein of the leaf may be a new type of combination of protein with carbohydrate-containing groups. However, it has not yet been established that the pentosan is actually combined, rather than admixed, with this protein.

Nucleic acid has not been detected in preparations of the colloidal protein. Their low phosphorus content shows that at the most the proportion of nucleic acid is small.

The colloidal protein is doubtless a mixture of several individual proteins which are constituents of the cytoplasm, protoplasts, and other elements of the cell, but owing to their insolubility in neutral solvents these cannot be separated from one another.

Apparently the colloidal protein occurs in the leaf, in chemical combination with chlorophyll, phosphatides, and probably other substances. This compound forms a colloidal solution with water which is very sensitive to the action of alcohol, being readily decomposed thereby into its component parts.

Spinach leaves dried at a low temperature and extracted with ether, alcohol, water, and alkaline solutions yield results so similar to those obtained with the fresh green leaf that evidently the constituents of the cells are altered to only a slight degree by drying.

DIGESTIBILITY OF RAW CORN, POTATO, AND WHEAT STARCHES.*

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INTRODUCTION.

It has been generally believed that raw starches are only digested a little, if at all, by the human body. Thorpe[1] quoting from Roberts says that starch in a raw state "is to man an almost indigestible substance, but when previously subjected to the operation of cooking it is digested with great facility." He further states that "diastase has, at best, only a comparatively feeble action on the unbroken starch granule, even at the temperature of the body."

Little experimental evidence is to be found regarding the digestibility of raw starches by the human body. Fofanow[2] conducted a series of experiments on human subjects with small quantities of raw wheat, oat, rice, and potato starches. He found that raw wheat, oat, and rice starches were practically completely assimilated, while raw potato starch was from two and one-half to four times less well digested. However, in his tests he used only 50 gm. of raw starch, a quantity which might easily be lost in the digestive tract. Numerous artificial digestion experiments have been conducted on raw starches which in general have demonstrated that raw starches are much more slowly acted on by the digestive ferments than starches which have been cooked.[3]

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[1] Thorpe, E., A dictionary of applied chemistry, London, New York, Bombay, and Calcutta, 1913, v, 179.

[2] Fofanow, L., *Z. klin. Med.*, 1911, lxxii, 257.

[3] Day, E. A., *U. S. Dept. Agric., Bull. 202*, 1908.

EXPERIMENTAL.

The methods in the experiments with corn, wheat, and potato starches were practically the same as in previous digestion experiments conducted by this office. In order that the starch should be in an appetizing form, it was eaten as a constituent of a frozen pudding. This was palatable and the subjects were able to eat it in fairly large quantities.

The frozen pudding contained approximately 20 per cent of raw starch and resembled ordinary ice-cream in taste and texture. This method of preparation did not affect the starch granules which were found by the Microchemical Laboratory of the Bureau of Chemistry to be neither swollen nor broken. The pudding was made as follows:

Experimental Frozen Pudding.

6 quarts milk.	2½ cups sugar.
4 pounds raw starch.	1 tablespoon salt.
3 cups table oil.	½ cup lemon extract.

The uncooked starch was mixed with milk, sugar, and oil in the proportions given above and the mixture immediately frozen in the same way as ice-cream. The lemon extract used for flavoring masked to a great extent the taste of uncooked starch and gave the frozen pudding a pleasing flavor.

The subjects were given weighed portions of the frozen pudding along with a basal ration of oranges and sugar. Tea or coffee was used if desired. The experiments were of 3 days or nine meals duration. The subjects were students in a local university who were apparently in normal health. They were familiar with this type of work, having served as subjects in previous experiments, and were entirely trustworthy. The methods for the separation of feces, analyses, *etc.* were those usually followed.

Corn-Starch.

Three experiments were conducted with raw corn-starch. The essential data for the interpretation of the results of these experiments are given in Tables I and II.

TABLE I.

Digestion Experiments with Uncooked Corn-Starch in a Simple Mixed Diet.

Experiment, subject, and diet.	Weight of food.	Constituents of foods.				
		Water.	Protein.	Fat.	Carbohydrate.	Ash.
Experiment No. 1047, Subject W. V. D.: Frozen custard containing uncooked starch (= 67 per cent total carbohydrate = 644 gm.), *gm.*	3,365.0	2,030.1	76.4	277.3	961.4	19.8
Fruit, *gm.*	818.0	710.8	6.6	1.6	94.9	4.1
Sugar, *gm.*	138.0				138.0	
Total food consumed, *gm.*	4,321.0	2,740.9	83.0	278.9	1,194.3	23.9
Feces, *gm.*	76.0		21.6	30.5	10.2	13.7
Amount utilized, *gm.*			61.4	248.4	1,184.1	10.2
Digestibility of entire ration, *per cent.*			74.0	89.1	99.1	42.7
Estimated digestibility of uncooked starch alone, *per cent.*					100.0	
Experiment No. 1048 Subject H. L. G.: Frozen custard containing uncooked starch (= 67 per cent total carbohydrate = 773 gm.), *gm.*	4,038.0	2,436.1	91.7	332.7	1,153.7	23.8
Fruit, *gm.*	1,193.0	1,036.7	9.5	2.4	138.4	6.0
Sugar, *gm.*	62.0				62.0	
Total food consumed, *gm.*	5,293.0	3,472.8	101.2	335.1	1,354.1	29.8
Feces, *gm.*	55.0		15.8	19.7	10.8	8.7
Amount utilized, *gm.*			85.4	315.4	1,343.3	21.1

TABLE I—*Concluded.*

Experiment, subject, and diet.	Weight of food.	Constituents of foods.				
		Water.	Protein.	Fat.	Carbohydrate.	Ash.
Digestibility of entire ration, *per cent*.....			84.4	94.1	99.2	70.8
Estimated digestibility of uncooked starch alone, *per cent*....					100.0	
Experiment No. 1049, Subject E. L.M.:						
Frozen custard containing uncooked starch (= 67 per cent total carbohydrate = 751 gm.), *gm*.....	3,924.0	2,367.3	89.1	323.3	1,121.1	23.2
Fruit, *gm*.....	1,127.0	979.4	9.0	2.3	130.7	5.6
Sugar, *gm*.....	31.0				31.0	
Total food consumed, *gm*.....	5,082.0	3,346.7	98.1	325.6	1,282.8	28.8
Feces, *gm*.....	51.0		14.8	14.9	15.0	6.3
Amount utilized, *gm*.....			83.3	310.7	1,267.8	22.5
Digestibility of entire ration, *per cent*.....			84.9	95.4	98.8	78.1
Estimated digestibility of uncooked starch alone, *per cent*...					100.0	
Average food consumed per subject per day, *gm*..	1,633.0	1,062.3	31.4	104.4	425.7	9.2

The diet as a whole supplied on an average 31 gm. of protein, 104 gm. of fat, 426 gm. of carbohydrate, and had an average energy value of 2,760 calories per man per day. The average amount of raw corn-starch eaten per man per day was 241 gm. Subject H. L. G. in Experiment 1048 ate the maximum amount of raw corn-starch which was 258 gm. per day for the experimental period.

The coefficient of digestibility of the raw corn-starch was found in each case to be 100 per cent after correction is made for

TABLE II.

Summary of Digestion Experiments with Uncooked Corn-Starch in a Simple Mixed Diet.

Experiment No.	Subject.	Digestibility of entire ration.				Estimated digestibility of uncooked corn-starch alone.
		Protein.	Fat.	Carbohydrate.	Ash.	
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
1047	W. V. D.	74.0	89.1	99.1	42.7	100.0
1048	H. L. G.	84.4	94.1	99.2	70.8	100.0
1049	E. L. M.	84.9	95.4	98.8	78.1	100.0
Average.....		81.1	92.9	99.0	63.9	100.0

the undigested residue from the accessory foods. This was confirmed by the Microchemical Laboratory of the Bureau of Chemistry which reported that no unruptured starch grains could be detected in the feces and that the iodine test for starch in the feces was negative.

The ingestion of this frozen custard caused no noticeable physiological effects and the subjects were in normal health during the experimental period. They reported the diet as appetizing and satisfying, a fact which would indicate that the starch was assimilated to a great extent by the body.

Potato Starch.

Seven experiments were conducted with potato starch—two in one test and five in a second test a few weeks later. The two subjects who took part in the first test had taken part in the experiments with raw corn-starch. In the second test with raw potato starch these men were joined by three others, one of whom had taken part in the experiments with raw corn-starch.

Tables III and IV are a summary of the results of the experiments with raw potato starch.

The diet as a whole furnished on an average 23 gm. of protein, 76 gm. of fat, 357 gm. of carbohydrate, and had an average energy value of 2,213 calories per man per day. The average amount of starch eaten per man per day was 194 gm.

It will be noted from Tables III and IV that the amount of potato starch digested varied considerably with the different subjects and even with the same subject in successive periods. Subject J. F. S. in Experiment 1082 showed the highest coefficient of digestibility (95.2 per cent) while Subject E. L. M. in Experiment 1081 showed the lowest (62.3 per cent). Subject H. L. G. in the first test ate 573 gm. of raw starch of which 74.5 per cent was digested. In the second test the digestibility increased to 85.4 per cent despite the fact that 120 gm. more of potato starch were eaten than in the first. On the other hand, Subject E. L. M. in the first test ate 503 gm. of raw potato starch of which 74.3 per cent was digested while in the second test only 62.3 per cent of the 710 gm. eaten was digested. There seemed to be no definite relation between the amount eaten and the amount digested. A possible explanation of the variation in the ability of the various subjects and of the same subjects in different periods to digest the raw potato starch may be that the body was unable to supply a large enough amount of amylases in a given time to digest all the raw starch.

During this diet practically all the subjects noted a very excessive formation of gas and frequent intestinal cramps. The quantity of feces voided was very large; a large amount of undigested starch was visible and a strong positive reaction was given with iodine. When the feces were ashed, the odor resembled that of scorched bread and no fecal odor was evident. The figure for the digestibility of protein is probably too low, since only a small amount was eaten and no correction has been made for metabolic nitrogen in the feces. The coefficient of digestibility for the cream and corn oil was 95.7 per cent. This agrees closely with previous determinations by this office which were 96.9[4] per cent for cream and 97.9[5] per cent for corn oil.

[4] Langworthy, C. F., and Holmes, A. D., *U. S. Dept. Agric., States Relations Service, Bull. 507*, 1917.

[5] Holmes, A. D., *U. S. Dept. Agric., States Relations Service, Bull. 687*, 1918.

TABLE III.

Digestion Experiments with Uncooked Potato Starch in a Simple Mixed Diet.

Experiment, subject, and diet.	Weight of food.	Constituents of foods.				
		Water.	Protein.	Fat.	Carbohydrate.	Ash.
Experiment No. 1062, Subject H. L. G.:						
Frozen custard containing 572.6 gm. uncooked starch, *gm.*	2,823.0	1,707.8	60.7	216.4	816.5	21.6
Fruit, *gm.*	913.0	793.4	7.3	1.8	105.9	4.6
Sugar, *gm.*	68.0				68.0	
Total food consumed, *gm.*	3,804.0	2,501.2	68.0	218.2	990.4	26.2
Feces, *gm.*	194.0		17.3	6.1	163.0	7.6
Amount utilized, *gm.*			50.7	212.1	827.4	18.6
Digestibility of entire ration, *per cent.*			74.6	97.2	83.4	71.0
Estimated digestibility of uncooked starch alone, *per cent.*					74.5	
Experiment No. 1063, Subject E. L. M.:						
Frozen custard containing 502.8 gm. uncooked starch, *gm.*	2,491.0	1,508.5	53.7	191.5	718.3	19.0
Fruit, *gm.*	725.0	630.0	5.8	1.5	84.1	3.6
Sugar, *gm.*	105.0				105.0	
Total food consumed, *gm.*	3,321.0	2,138.5	59.5	193.0	907.4	22.6
Feces, *gm.*	191.0		27.8	10.7	143.9	8.6
Amount utilized, *gm.*			31.7	182.3	763.5	14.0
Digestibility of entire ration, *per cent.*			53.3	94.5	84.1	61.9
Estimated digestibility of uncooked starch alone, *per cent.*					74.3	

TABLE III—*Continued.*

Experiment, subject, and diet.	Weight of food.	Constituents of foods.				
		Water.	Protein.	Fat	Carbohydrate.	Ash.
Experiment No. 1078, Subject P. C.: Frozen custard containing 467.7 gm. uncooked starch, *gm.*	2,505.0	1,553.1	51.6	184.6	698.2	17.5
Fruit, *gm.*	1,372.0	1,192.3	11.0	2.7	159.1	6.9
Sugar, *gm.*	183.0				183.0	
Total food consumed, *gm.*	4,060.0	2,745.4	62.6	187.3	1,040.3	24.4
Feces, *gm.*	243.0		28.9	11.0	191.4	11.7
Amount utilized, *gm.*			33.7	176.3	848.9	12.7
Digestibility of entire ration, *per cent*			53.8	94.1	81.6	52.0
Estimated digestibility of uncooked starch alone, *per cent*					64.3	
Experiment No. 1079, Subject W. V. D.: Frozen custard containing 544.8 gm. uncooked starch, *gm.*	2,918.0	1,809.2	60.1	215.1	813.2	20.4
Fruit, *gm.*	807.0	701.3	6.5	1.6	93.6	4.0
Sugar, *gm.*	97.0				97.0	
Total food consumed, *gm.*	3,822.0	2,510.5	66.6	216.7	1,003.8	24.4
Feces, *gm.*	105.0		21.9	10.8	64.1	8.2
Amount utilized, *gm.*			44.7	205.9	939.7	16.2
Digestibility of entire ration, *per cent*			67.1	95.0	93.6	66.0
Estimated digestibility of uncooked starch alone, *per cent*					91.3	

TABLE III—*Continued.*

Experiment, subject, and diet.	Weight of food.	Constituents of foods.				
		Water.	Protein.	Fat.	Carbohydrate.	Ash.
Experiment No. 1080, Subject H. L. G.: Frozen custard containing 691.3 gm. uncooked starch, *gm.*	3,703.0	2,295.9	76.3	272.9	1,032.0	25.9
Fruit, *gm.*	731.0	635.2	5.8	1.5	84.8	3.7
Sugar, *gm.*	43.0				43.0	
Total food consumed, *gm.*	4,477.0	2,931.1	82.1	274.4	1,159.8	29.6
Feces, *gm.*	149.0		18.2	7.3	117.4	6.1
Amount utilized, *gm.*			63.9	267.1	1,042.4	23.5
Digestibility of entire ration, *per cent*			77.8	97.3	89.9	79.2
Estimated digestibility of uncooked starch alone, *per cent*					85.4	
Experiment No. 1081, Subject E. L. M.: Frozen custard containing 710.0 gm. uncooked starch, *gm.*	3,803.0	2,357.8	78.3	280.3	1,059.9	26.7
Fruit, *gm.*	526.0	457.1	4.2	1.1	61.0	2.6
Sugar, *gm.*	105.0				105.0	
Total food consumed, *gm.*	4,434.0	2,814.9	82.5	281.4	1,225.9	29.3
Feces, *gm.*	332.0		27.4	11.6	283.2	9.8
Amount utilized, *gm.*			55.1	269.8	942.7	19.5
Digestibility of entire ration, *per cent*			66.8	95.9	76.9	66.6
Estimated digestibility of uncooked starch alone, *per cent*					62.3	

TABLE III—*Concluded.*

Experiment, subject, and diet.	Weight of food.	Constituents of foods				
		Water.	Protein.	Fat.	Carbohydrate.	Ash.
Experiment No. 1082, Subject J. F. S.:						
Frozen custard containing 580.1 gm. uncooked starch, *gm.*	3,107.0	1,926.3	64.0	229.0	865.9	21.8
Fruit, *gm.*	1,324.0	1,150.6	10.6	2.6	153.6	6.6
Sugar, *gm.*	166.0				166.0	
Total food consumed, *gm.*	4,597.0	3,076.9	74.6	231.6	1,185.5	28.4
Feces, *gm.*	100.0		24.3	8.9	52.1	14.7
Amount utilized, *gm.*			50.3	222.7	1,133.4	13.7
Digestibility of entire ration, *per cent*			67.4	96.2	95.6	48.1
Estimated digestibility of uncooked starch alone, *per cent*					95.2	
Average food consumed per subject per day, *gm.*	1,357.9	891.4	23.6	76.3	357.8	8.8

TABLE IV.

Summary of Digestion Experiments with Uncooked Potato Starch in a Simple Mixed Diet.

Experiment No.	Subject.	Digestibility of entire ration.				Estimated digestibility of uncooked potato starch alone.
		Protein.	Fat.	Carbohydrate.	Ash.	
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
1062	H. L. G.	74.6	97.2	83.4	71.0	74.5
1063	E. L. M.	53.3	94.5	84.1	61.9	74.3
1078	P. C.	53.8	94.1	81.6	52.0	64.3
1079	W. V. D.	67.1	95.0	93.6	66.0	91.3
1080	H. L. G.	77.8	97.3	89.9	79.2	85.4
1081	E. L. M.	66.8	95.9	76.9	66.6	62.3
1082	J. F. S.	67.4	96.2	95.6	48.1	95.2
Average		65.8	95.7	86.4	63.5	78.2

TABLE V.

Digestion Experiments with Uncooked Wheat Starch in a Simple Mixed Diet.

Experiment, subject, and diet.	Weight of food.	Constituents of foods.				
		Water.	Protein.	Fat.	Carbohydrate.	Ash.
Experiment No. 1073, Subject P. C.: Frozen custard containing 462.4 gm. uncooked starch, *gm.*	2,493.0	1,573.3	48.4	164.8	690.3	16.2
Fruit, *gm.*	728.0	632.6	5.8	1.5	84.5	3.6
Sugar, *gm.*	120.0				120.0	
Total food consumed, *gm.*	3,341.0	2,205.9	54.2	166.3	894.8	19.8
Feces, *gm.*	31.0		5.1	5.7	16.5	3.7
Amount utilized, *gm.*			49.1	160.6	878.3	16.1
Digestibility of entire ration, *per cent*			90.6	96.6	98.2	81.3
Estimated digestibility of uncooked starch alone, *per cent*					99.8	
Experiment No. 1075, Subject H. L. G.: Frozen custard containing 692.7 gm. uncooked starch, *gm.*	3,734.0	2,356.5	72.4	246.8	1,034.0	24.3
Fruit, *gm.*	597.0	518.8	4.8	1.2	69.2	3.0
Sugar, *gm.*	8.0				8.0	
Total food consumed, *gm.*	4,339.0	2,875.3	77.2	248.0	1,111.2	27.3
Feces, *gm.*	56.0		4.5	17.9	23.4	10.2
Amount utilized, *gm.*			72.7	230.1	1,087.8	17.1
Digestibility of entire ration, *per cent*			94.2	92.8	97.9	62.6
Estimated digestibility of uncooked starch alone, *per cent*					98.6	

TABLE V—*Concluded.*

Experiment, subject, and diet.	Weight of food.	Constituents of foods.				
		Water.	Protein.	Fat.	Carbohydrate.	Ash.
Experiment No. 1076, Subject E. L. M.:						
Frozen custard containing 597.9 gm. uncooked starch, *gm.*	3,223.0	2,034.0	62.5	213.0	892.5	21.0
Fruit, *gm.*	1,294.0	1,124.5	10.3	2.6	150.1	6.5
Sugar, *gm.*	201.0				201.0	
Total food consumed, *gm.*	4,718.0	3,158.5	72.8	215.6	1,243.6	27.5
Feces, *gm.*	68.0		6.1	17.6	33.5	10.8
Amount utilized, *gm.*			66.7	198.0	1,210.1	16.7
Digestibility of entire ration, *per cent.*			91.6	91.8	97.3	60.7
Estimated digestibility of uncooked starch alone, *per cent.*					98.4	
Experiment No. 1077, Subject J. F. S.:						
Frozen custard containing 508.9 gm. uncooked starch, *gm.*	2,743.0	1,731.1	53.2	181.3	759.6	17.8
Fruit, *gm.*	1,115.0	968.9	8.9	2.2	129.4	5.6
Sugar, *gm.*	172.0				172.0	
Total food consumed, *gm.*	4,030.0	2,700.0	62.1	183.5	1,061.0	23.4
Feces, *gm.*	22.0		4.3	10.5	5.0	2.2
Amount utilized, *gm.*			57.8	173.0	1,056.0	21.2
Digestibility of entire ration, *per cent.*			93.1	94.3	99.5	90.6
Estimated digestibility of uncooked starch alone, *per cent.*					100.0	
Average food consumed per subject per day, *gm.*	1,369.0	911.6	22.2	67.8	359.2	8.2

TABLE VI.

Summary of Digestion Experiments with Uncooked Wheat Starch in a Simple Mixed Diet.

Experiment No.	Subject.	Digestibility of entire ration.				Estimated digestibility of uncooked wheat starch alone.
		Protein.	Fat.	Carbohydrate.	Ash.	
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
1073	P. C.	90.6	96.6	98.2	81.3	99.8
1075	H. L. G.	94.2	92.8	97.9	62.6	98.6
1076	E. L. M.	91.6	91.8	97.3	60.7	98.4
1077	J. F. S.	93.1	94.3	99.5	90.6	100.0
Average		92.4	93.9	98.2	73.8	99.2

Wheat Starch.

Four experiments were conducted to determine the digestibility of raw wheat starch. All four of the subjects had taken part in the tests with raw potato starch, and two in the earlier ones with raw corn-starch.

The essential data for the interpretation of the experiments are given in Tables V and VI.

The diet as a whole furnished an average of 22 gm. of protein, 68 gm. of fat, and 359 gm. of carbohydrate per man per day with an average fuel value of 2,138 calories. The average amount of raw starch eaten per man per day was 188 gm. The subjects remained in apparently normal health during the diet period. It will be noted that practically all the wheat starch was assimilated in each case. No starch could be detected in the feces by the iodine test. Moreover, the other constituents were very well assimilated, the coefficients of digestibility being 92 per cent for protein and 94 per cent for fat.

SUMMARY.

1. Raw corn and wheat starches were found to be completely assimilated and no trace of them could be found in the feces.

2. Seven experiments on raw potato starch gave values for its digestibility varying from 62.3 to 95.2 per cent; the average was 78.2 per cent.

3. The ingestion of the potato starch caused disagreeable physiological disturbances not noted in the other experiments with raw corn and wheat starches.

4. The digestibility of the other constituents of the diet was not affected to any great extent by the large amounts of raw starch ingested.

THE CO_2 CONTENT AS A BASIS FOR DISTINGUISHING HEATED FROM UNHEATED MILK.

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INTRODUCTION.

It has been pointed out in a previous article[1] that the heating of milk, when pasteurized, reduces the CO_2 content to such an extent that this change might be made the basis of a method for distinguishing pasteurized from unheated milk. In order to ascertain whether this fact of decrease of CO_2 could be utilized in the development of such a method, it is necessary to learn to what extent the CO_2 content of normal milk is reduced under a variety of conditions, especially such conditions as are associated with the handling of milk from the time it leaves the udder until it reaches the consumer. A study has, therefore, been made of the effect of such conditions as (1) the method of milking, (2) the length of time of standing of milk after being drawn from the udder, (3) the effect of agitation, and (4) the effect of temperature. The results of this work are presented in the pages following.

The details of the method used in the determination of CO_2 in milk are explained in the article already referred to.

EXPERIMENTAL.

When milk is drawn from the udder and exposed to the air, a considerable proportion of its CO_2 escapes. The amount of CO_2 usually present in normal milk in the udder has been found in the former work, to which reference has already been made, to

[1] Van Slyke, L. L., and Baker, J. C., *J. Biol. Chem.*, 1919, xl, 335.

average about 10 per cent by volume. As a result of our work here given, we find that immediately after milk is drawn from the udder the CO_2 content is generally less than one-half of this figure, varying between 4 and 5 per cent in most cases.

It is our purpose now to give the detailed results of our work, showing to what extent different conditions affect the CO_2 content of milk after leaving the udder.

1. Effect of Method of Milking.

A few determinations of CO_2 were made in the case of milk drawn from the udder by hand, in comparison with that drawn by milking-machine, two different kinds of machines being used. No appreciable difference was found in all the samples examined by us, the results varying only between 4 and 4.5 per cent of CO_2 by volume, without reference to the method of milking.

2. Effect of Standing.

Numerous examinations have been made by us to ascertain the amount of CO_2 in milk at different intervals after milking, when kept under ordinary conditions. Milk from individual cows and also from herds was used. After being drawn from the udder, the milk was kept under conditions involving a minimum amount of handling or agitation.

In the case of samples of herd milk, obtained at a local milk-station, the milk had been transported several miles directly from the farms to the station. The transportation was, of course, accompanied by more or less agitation. We examined forty such samples at intervals of 8, 20, and, in some cases, 40 hours after milking. The results are summarized in Table I.

These results indicate that, under the conditions existing, the volume per cent of CO_2 in no case falls below 3, even 40 hours after milking, and only in a few cases below 3.5 after 20 hours or more.

In the case of milk drawn separately from five individual cows in our station herd and examined, after standing without further handling, at intervals of 18, 42, 64, and 90 hours, the amount of CO_2 present in the milk soon after being drawn decreased by

0.5 to 1.0 per cent by volume in 18 hours, and 1.0 to 1.5 in 42 hours, after which there was no further decrease. The percentage of CO_2 in the fresh milk varied in the different samples from 4 to 4.5 and this dropped to a minimum of 3, no further decrease taking place after 42 hours.

In the case of the mixed milk of our station herd, which contained 4 per cent by volume of CO_2 3 or 4 hours after milking, it was found after standing 6 hours more to contain 3.5 per cent, a decrease of 0.5 per cent.

TABLE I.

CO_2 in Herd Milk at Intervals after Milking.

CO_2 in samples.	No. of samples examined after		
	8 hrs.	20 hrs.	49 hrs.
vol. per cent			
3.0	0	3	2
3.5	12	5	3
4.0	16	17	6
4.5	7	9	2
5.0	3	4	1
5.5	2	2	0

3. Effect of Agitation.

Several experiments were made to ascertain the effect of agitation in different forms upon the CO_2 content of milk. Milk was passed through a centrifugal separator in some cases and in others was stirred by special apparatus.

In the case of milk passed through a cream-separator, the milk before separation contained 4 per cent of CO_2 by volume. The resulting skim-milk was found to contain the same amount of CO_2, while the cream contained 3 per cent. In the case of another sample of milk, containing 3.5 per cent of CO_2 by volume before separation, the resulting skim-milk contained the same percentage of CO_2. Skim-milk containing 4 per cent of CO_2 by volume was found after being run through a separator to lose no CO_2.

In experiments in which the milk was stirred, the agitation was produced by means of the stirring-apparatus described in a former article.[2]

[2] Van Slyke, L. L., and Baker, J. C., *J. Biol. Chem.*, 1918, xxxv, 127.

In the case of a sample of milk containing 4 per cent of CO_2 by volume, the milk was kept at room temperature (about 21°C.) during the stirring, and examinations were made at stated intervals, with the following results:

Length of time of stirring	0	15 min.	30 min.	45 min.	1 hr.	2 hrs.	6 hrs.
Percentage of CO_2 by volume	4	3.5	3	3	3	2.5	2

From these results it appears that such agitation as milk is subjected to in the methods commonly employed in handling milk on farms and before being placed on the market has little or no effect in reducing the percentage of CO_2.

4. Effect of Heating.

Experiments were made for the purpose of finding out the effect of increased temperature upon the CO_2 content of milk, using different temperatures, and also stirring at the same time in some cases. The special object in mind was to learn the effect of those conditions commonly employed in pasteurizing milk.

In the case of milk pasteurized by the "flash" system, samples containing 4 per cent of CO_2 by volume were found after pasteurization to contain not more than 2 per cent.

Numerous samples of market milk, pasteurized at about 62°C. (143°F.) and bottled, were examined 30 to 36 hours after pasteurization and found to contain 1.5 to 2 per cent of CO_2 by volume.

Milk heated at 62°C. (143°F.) for 30 minutes was examined before and after cooling to 5.5°C. (42°F.). The amount of CO_2 was found to be the same in both cases, 2 per cent.

Fresh milk containing 4 per cent of CO_2 by volume was heated at about 63°C. (145°F.) without agitation for different periods of time, the following results being obtained:

Minutes heated	2	3	4	5	10	12	20	30
Percentage of CO_2 by volume	3.5	3	2.5	2.5	2.5	2.5	2.5	2.5

A sample of milk heated to about 78°C. (172°F.), without stirring, for 30 minutes contained 2.5 per cent of CO_2 by volume. Another sample heated for 30 minutes to about 62°C. (143°F.) with stirring was found to contain no CO_2.

Milk, pasteurized by the methods commonly employed, and as found by us in the market, seldom contains more than 2.5 per cent of CO_2 by volume and usually contains less.

Distinction between Heated and Unheated Milk.

Taking into consideration all the results of our work, it appears that, under the conditions to which normal unheated milk is subjected in its handling from the time of milking to the time of delivery to the consumer, the volume percentage of CO_2 rarely, if ever, drops below 3 and seldom below 3.5; while the subjection of normal milk to the conditions of heating used in pasteurization reduces the percentage of CO_2 by volume to 2.5 or less. Therefore, it appears safe, in general, to assume that milk containing less than 2.5 or 3 per cent of CO_2 by volume has been heated to the temperature of pasteurization.

A STUDY OF THE DISTRIBUTION OF IODINE BETWEEN CELLS AND COLLOID IN THE THYROID GLAND.

I. METHODS AND RESULTS OF STUDY OF BEEF, SHEEP, AND PIG THYROID GLANDS.

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(Received for publication, March 8, 1920.)

At the present time there seems fairly general agreement among investigators that iodine in the thyroid gland plays a significant rôle in thyroid function. As a matter of fact most biochemical and physiological studies of thyroid gland activity have centered about iodine which has been taken as the basis of potency of preparations and a presumptive index of the state of the gland.

Morphological studies have partially correlated the architectural characteristics with iodine content of the gland. Oswald,[1] Kocher,[2] Marine and Williams,[3] and others have found a fair parallelism between the quantity of colloid and the quantity of iodine of approximately normal glands. It must be remembered, however, that the estimation of the amount and concentration of colloid in any specimen of gland has not as yet been placed on a satisfactory basis, consequently the concentration of iodine in the colloid material cannot be accurately judged. On the other hand more evidently extreme colloid and hyperplastic types of glands appear to be anomalous in this relation which according to Kocher[2] is due to variations in concentration of colloid material.

Concerning the relative significance of colloid to glandular activity Bensley[4] brought out morphological evidences based on

[1] Oswald, A., *Virchows Arch. path. Anat.*, 1902, clxix, 444.
[2] Kocher, A., *Virchows Arch. path. Anat.*, 1912, ccviii, 86.
[3] Marine, D., and Williams, W. W., *Arch. Int. Med.*, 1908, i, 349.
[4] Bensley, R. R., *Am. J. Anat.*, 1916, xix, 37.

selective staining reactions indicating that the active secretion of the thyroid gland passes through what is usually considered the bases of the cells into blood or lymphatics and that the visible colloid represents either a waste product or a reserve. Whether colloid is considered as a reserve or as a waste product the mode of its quantitative diminution or access to the circulation is yet an unsolved problem.

One great difficulty in evaluating the relative importance and significance of cells *versus* colloid, the latter being usually considered the result of active secretion of the gland, has been the inability to locate and follow accurately the course of the iodine-containing body within the gland itself. Most investigators believe, following Baumann[5] and Oswald,[6] that the colloid is a secretion and is the material in which iodine is to be found. Oswald,[6] it will be recalled, scraped out mechanically colloid material from colloid-rich glands fixed in 70 per cent alcohol and found iodine. The iodine content of the cells of colloid-containing glands had not been specifically determined.[7]

As a consequence, it is highly desirable to apply a method adequate to locate the iodine within the gland in order to determine whether it is all in the colloid, all in the cells, or whether there exists a physiological equilibrium between the iodine of the colloid and the iodine of the cells, and further if there exists such an equilibrium it is desirable to determine the controlling factors. It is by such a study that evidences may be obtained bearing on the physical chemistry of the gland and the relative significance of colloid and active cells. Further, such knowledge might likely have an immediate bearing upon our conceptions of the mode of secretion and character of action of the normal stimulants to the gland whatever they may subsequently be found to be. It is with this basic problem of localization of iodine-containing constituents in the thyroid gland that the work here presented is concerned.

[5] Baumann, E., *Z. physiol. Chem.*, 1895–96, xxi, 319; 1896–97, xxii, 1.
[6] Oswald, A., *Z. physiol. Chem.*, 1899, xxvii, 14.
[7] Claude, H., and Blanchetière, A., *J. physiol. et path. gén.*, 1910, xii. 563.

Methods.

Separation of Cells from Colloid.

As is perhaps generally known to morphologists frozen section preparations of unfixed thyroid glands do not retain the colloid material when such sections are floated in isotonic salt solution. It is evident then that in such preparations the fluid colloid readily leaves the opened acini. I have repeatedly tried to obtain evidences of colloid by selective staining reactions in such sections and have always failed. Furthermore when the frozen preparations were prepared in an ammonia-cooled room, the completely frozen section being placed immediately upon a slide, it was observed that after bringing the slide outside the cold chamber into a room at ordinary room temperature the colloid was readily demonstrated to have spread more or less uniformly over the whole section as would glycerol or other fluid. These facts led to the idea of utilizing such preparations as a means of separating cells from colloid for purposes of chemical analysis.

Thyroid glands obtained from the abattoir[8] or the laboratory as soon as possible after death of the animals were cut into blocks of suitable size for cutting on the freezing microtome. Sections were cut as thin as efficiently possible, or at least sufficiently thin to open all acini. Such sections were placed in Ringer's solution immediately from the microtome knife, the sections separated from one another, and straightened out. After a sufficient quantity of sectioned material was obtained the sections were either picked out with a dissecting needle or, as a routine procedure, the whole mixture was centrifuged directly. The liquor was poured off (in some instances this liquor itself was analyzed and found to contain iodine), then a second portion of salt solution added to the cell mass, and the whole thoroughly stirred in order to remove any adhering colloid material. This second mixture was centrifuged, the liquor poured off, and then the cell mass, including unavoidable stroma, or connective tissue, washed out with a small quantity of distilled water onto a watch-glass. The material was evaporated carefully over a hot plate and then finally dried at 105°C. Unsectioned specimens of the whole gland were dried in the same manner for control values.

[8] Through the courtesy of Armour and Company, Chicago.

TABLE I.

Quantitative Determination of Iodine in Whole Glands and of Cells Free from Colloid Material.

Animal No.	Weight of whole gland used.	Iodine in whole gland.	Weight of cell mass used.	Iodine in cell mass.	Ratio of percentage of iodine in cells to percentage of iodine in whole gland.	Morphology.	
						Cells.	Colloid.
	mg.	*per cent*	*mg.*	*per cent*			
Pig 1	250.2	0.377	171.5	0.076	0.20	Flat.	Rich.
" 2	222.6	0.81	127.2	0.274	0.34	"	"
Sheep 1	251.0	0.26	104.0	0.096	0.37	"	"
" 2	367.0	0.442	126.5	0.145	0.33	"	"
" 3	266.5	0.265	125.5	0.076	0.28	"	"
" 4	297.0	0.198	139.5	0.046	0.23	"	"
" 5	364.0	0.19	111.0	0.078	0.41	"	"
" 6	334.0	0.273	112.0	0.086	0.31	"	"
" 7	258.5	0.165	93.0	0.065	0.39	"	"
" 8	308.5	0.089	195.0	0.027	0.30	Cuboidal.	Fair.
" 9	231.0	0.129	90.3	0.048	0.37	Flat.	Rich.
Beef 1	288.0	0.151	177.0	0.128	0.85*	"	"
" 2	248.0	0.025	155.5	0.011	0.44	Columnar.	Poor.
" 3	184.0	0.308	153.7	0.128	0.42	Flat.	Rich.
" 4	166.0	0.468	145.0	0.192	0.41	"	"
" 5	141.0	0.409	163.0	0.118	0.29	"	"
" 6	263.0	0.429	148.0	0.118	0.28	"	"
" 7	145.0	0.021	111.5	None.		Columnar.	Poor.
" 8	228.5	0.103	80.5	0.054	0.53*	"	"
" 9	240.5	0.065	186.5	0.055	0.86*	"	"
" 10	134.5	0.023	118.3	0.011	0.48	"	"
" 11	200.5	0.043	182.5	0.009	0.21	"	"
" 12	207.0	0.042	113.5	0.015	0.36	"	"
" 13	252.2	0.039	249.5	0.014	0.37	"	"
" 14	202.8	0.366	165.5	0.111	0.30	Flat.	Rich.
" 15	248.9	0.040	215.5	0.010	0.25	Columnar.	Poor.
" 16	343.5	0.051	220.8	0.020	0.39	"	"
" 17	349.3	0.005	319.3	0.0054	1.08†	"	"
" 18	302.0	0.095	191.5	0.041	0.43	"	"

* Unexplainable high values.

† Cells appear to contain a higher concentration of iodine than whole gland, though the actual concentration is too low to be accurately determinable in such amounts of whole gland and of cell material used.

Iodine Determination.

The method used for iodine determination was that described by Kendall.[9] This method was checked prior to adoption, by analysis of dried thyroid gland previously analyzed by the Hunter method, and was found to be satisfactory for such material containing measurable quantities of iodine or quantities of material containing as a minimum 0.008 mg. of iodine. The limits of sensitivity appear to be rather the limits of thiosulfate titration than the preparatory technique.

The actual concentration of iodine in thyroid cells is evidently higher than the values given in Table I for the reason that not alone epithelial cells but also connective tissues within and about the gland are included in the cell mass examined for iodine. However, the same criticism applies as well to the whole gland for the iodine concentration is based on total gross weight of the material analyzed.

DISCUSSION.

It will be seen from Table I that, whereas the individual percentages of iodine in different whole glands vary, the cells, with the exception of those from one gland, all contain measurable amounts of iodine, and furthermore that the *ratio of the percentage of iodine in cells to percentage of iodine in the whole gland comes to a fairly constant value.*

It is true that within the series of ratios themselves as much as 100 per cent variation occurs, yet they are nevertheless of the same order of magnitude and hence may be considered a physiological constant for glands in this particular functional condition. The values of the ratios appear to fall well within relatively narrow limits, regardless of the percentage values of the iodine in whole glands which differ among themselves up to 1,000 per cent. The wide variation of total iodine values adds considerable weight to the evidence of a relative percentage constant. While in the table of twenty-nine analyzed glands there are four examples in which high ratios were obtained, it does not seem likely that the remaining twenty-four fall between the limits of 0.20 and 0.48 by mere coincidence but are rather of some basic signifi-

[9] Kendall, E. C., *J. Biol. Chem.*, 1914, xix, 251.

cance. An occasional high value may be expected since in the method of separation adopted it is possible to have some unopened acini or incomplete separation or other unavoidable errors in technique. We feel that the lower values are more likely to be more exact. All available data have been presented without reservation, so that the case presented may stand on its own merits.

The observed relative percentage constant may be quite analogous to the partition coefficient of a substance distributed between the solvents in a diphasic system. On the other hand this same percentage constant need not necessarily hold for pathological states within the gland which may conceivably alter one or both phases or even the solute itself (iodine-containing molecule). Seasonal variation, various stages in physiological activity, or any other unknown factors might possibly alter the ratio though such does not appear to be evident from the series presented. These problems, however, are now being further investigated and will be reported in subsequent papers of this series of studies.

In regard to the dog thyroid glands, previously reported in abstract,[10] it may be stated that by using as a criterion the ratio values found for other species, the iodine values of the cells of such amounts of glands available from one animal calculate close to or below the lowest limits of sensitivity of the method for iodine determination. This explanation will rectify the conclusion recorded in the preliminary notice in which there did not appear to be any measureable iodine in the cell fraction. It is only rational then to suppose that by taking glands from several animals that a sufficient quantity of cells could be obtained so that quantitative studies of iodine content could be accurately made. This question will be more fully reported in a subsequent paper. It may be now stated, however, that by taking a sufficient quantity of cell material iodine is usually to be found though in very low concentration.[11]

It is desirable to add that the conclusions drawn herewith depend upon the safety of the method. As to the question of the effects of freezing upon diffusibility of iodine-containing compounds, whether or not under such a procedure the iodized mole-

[10] Tatum, A. L., *Proc. Soc. Exp. Biol. and Med.*, 1919, xvii, 28.

[11] From part of the work now in progress in this laboratory in collaboration with Mr. H. B. Van Dyke.

cule more readily leaves the cells or on the other hand enters the cells, the following evidences may be cited. Subsequent fixation and staining are inadequate to demonstrate any readily detectable alterations in morphology. Concerning diffusion changes it has been found that cells from iodine-poor glands placed in colloid-containing liquor obtained from iodine-rich glands do not appear to alter in iodine content within an hour or so at room temperature.[11] It must be admitted, however, that the iodine concentration of the liquor (Ringer's solution plus dissolved colloid) is far lower than the original colloid bathing the cells *in situ*. Yet this seems to us to answer possible objections on the basis of our measurements being a question of mere equilibria between cells in suspension and iodine of the colloid in solution. Since the liquor was relatively rich in organically bound iodine, cells poor in iodine should on a purely physical basis have been raised in iodine content to a value equal to that of the cells from the gland rich in iodine. But such was not the case. The iodine content of cells from the iodine-poor gland remained the same whether placed in iodine-rich or iodine-poor liquor.

SUMMARY.

1. A new method is described for the separation of cells from colloid of the thyroid gland for chemical study.

2. Iodine is found in both cells and colloid of beef, sheep,and pig thyroid glands.

3. The ratio of percentages of iodine in cells to iodine in whole glands appears to be relatively constant for pig, sheep, and beef thyroid glands, even though such glands vary both in morphology and in total iodine content.

FURTHER STUDIES ON THE ELIMINATION OF TAURINE ADMINISTERED TO MAN.*

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(Received for publication, March 3, 1920.)

The question as to the fate of certain amino-acids when given in large doses has been the subject of numerous papers. The evidence that the portion which is excreted by the kidney is combined with urea in the form of uramino-acid rests on the basis that this compound has been isolated from urine.

Schultzen [1] isolated sarcosincarbamic acid from the urine of a dog fed with sarcosin, but the quantitative differences in yield noted by Baumann and von Mering,[2] Salkowski,[3] and Schiffer [4] on repeating this experiment can hardly be attributed to individual variability of the experimental animals. The ease with which amino-acids combine with urea to form the corresponding uramino-acid [5] would lead one to suspect that the latter when found in urine was not preformed in the body but a resultant of the process used for its isolation, and the quantity obtained dependent upon the particular technique used. Dakin[6] fed inactive tyrosine and phenylalanine to cats and although he found, in accordance with results previously obtained by Blendermann,[7] uramino-acids in the urines of his experimental animals he recognizes the possibility that these substances were formed in the process of isolation from the unchanged amino-acid present in the urine. When the urine was acidified with acetic acid as soon as passed the yield of uramino-acid was so small as to be insufficient for identifica-

* Aided in part by a grant from the George Williams Hooper Foundation for Medical Research.

[1] Schultzen, O., *Ber. chem. Ges.*, 1872, v, 578.

[2] Baumann, E., and von Mering, J., *Ber. chem. Ges.*, 1875, viii, 584.

[3] Salkowski, E., *Z. physiol. Chem.*, 1880, iv, 100.

[4] Schiffer, J., *Z. physiol. Chem.*, 1881, v, 257.

[5] Weiland, W., *Biochem. Z.*, 1912, xxxviii, 385. Lippich, F., *Ber. chem., Ges.* 1908, xli, 2974.

[6] Dakin, H. D., *J. Biol. Chem.*, 1910, viii, 25, 35.

[7] Blendermann, H., *Z. physiol. Chem.*, 1882, vi, 234.

tion. The failure of Rohde[8] to obtain by analytical methods, after decomposing the urea with urease, evidence of the presence of uramino-acid in the urine of a cat injected with phenylalanine, indicates that the α-ureidio-β-phenylpropionic acid isolated by Dakin[9] in a similar experiment was formed after the urine had been passed.

The experiments of Salkowski[10] on the elimination of taurine have been of particular interest to us. He found that taurine when taken by mouth was eliminated in large part in the urine. This was determined by estimating the increase in neutral sulfur. The substance which he isolated was not taurine but taurocarbamic acid. Experiments reported by Schmidt, von Adelung, and Watson[11] confirmed Salkowski's findings with regard to the increase of neutral sulfur. It, however, appears to us in view of the doubt which exists as to the formation of uramino-acids in the body that the taurocarbamic acid isolated by Salkowski was formed after the urine had been passed. Our data presented below show that taurine is excreted in the free state and not combined with urea as taurocarbamic acid.

Since taurine yields its nitrogen quantitatively in 4 minutes[12] when shaken with nitrous acid while the uramino-acid gives off its nitrogen but slowly, use was made of the method of Van Slyke[13] for the determination of α-amino nitrogen and the nitrogen due to slowly reacting amines in the urine of a subject to whom taurine was administered. If taurine is excreted in the urine in the free state an increase in α-amino nitrogen over the normal output should be noted, but if it is present as taurocarbamic acid the nitrogen obtained from the slowly reacting amines should show a corresponding increase. Preliminary experiments in which taurine and taurocarbamic acid were added to urine showed that taurocarbamic acid was not split by urease,[14] and that the added taurine could be quantitatively estimated from the increase of α-amino nitrogen.

A subject was placed on a constant diet and the normal excretion of total nitrogen, amino-acid nitrogen, and sulfur determined.

[8] Rohde, A., *J. Biol. Chem.*, 1918, xxxvi, 467.

[9] Dakin, H. D., *J. Biol. Chem.*, 1909, vi, 235.

[10] Salkowski, E., *Virchows Arch. path. Anat.*, 1873, lviii, 460, 580; *Ber. chem. Ges.*, 1872, v, 637.

[11] Schmidt, C. L. A., von Adelung, E., and Watson, T., *J. Biol. Chem.*, 1918, xxxiii, 501.

[12] Foster, M. G., and Hooper, C. W., *J. Biol. Chem.*, 1919, xxxviii, 355.

[13] Van Slyke, D. D., *J. Biol. Chem.*, 1913-14, xvi, 125.

[14] Previously noted by Mateer, J. G., and Marshall, E. K., Jr., *J. Biol. Chem.*, 1916, xxv, 297.

TABLE I.

Subject I.

Day of experiment.	Total nitrogen.	α-amino nitrogen.	Nitrogen from slowly reacting amines.*	Total sulfur.	Total sulfates.	Neutral sulfur.	Remarks.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
1	10.1	0.38	0.11	0.80	0.66	0.14	
2	10.3	0.44	0.05	0.82	0.67	0.15	
3	9.3	0.38	0.10	0.72	0.58	0.14	
4	11.0	1.12	0.12	2.49	0.70	1.79	10 gm. taurine by mouth. S = 2.56 gm.
5	9.8	0.46	0.09	0.84	0.63	0.21	
6	9.3	0.38	0.09	0.78	0.56	0.22	

* Calculated on the basis of 4 minutes.

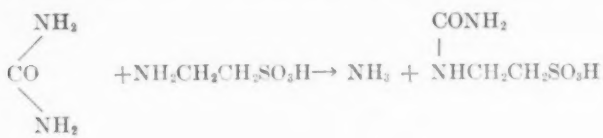
TABLE II.

Subject II.

Day of experiment.	Total nitrogen.	Urea nitrogen.	Urea nitrogen in total nitrogen.	Ammonia nitrogen.	Ammonia nitrogen in total nitrogen.	Remarks.
Experiment A.						
	gm.	*gm.*	*per cent*	*gm.*	*per cent*	
1	9.0	7.4	82	0.58	6.4	
2	9.4	7.4	79	0.54	5.7	
3	8.7	6.7	77	0.66	7.6	3.3 gm. taurine by mouth in 10 (hourly) doses.
4	9.4	7.3	78	0.57	6.1	
5	10.4	7.3	70	0.79	7.6	14 gm. taurine by mouth in 10 (hourly) doses.
6	9.3	7.4	80	0.65	7.0	
Experiment B.						
1	6.6	5.7	86	0.44	6.7	
2	7.5	6.2	83	0.52	6.9	
3	8.7	7.0	80	0.51	5.9	
4	9.5	7.0	74	0.48	5.1	15 gm. taurine intravenously.
5	8.2	7.2	88	0.43	5.2	
6	8.8	6.8	77	0.46	5.2	15 gm. taurine intravenously.
7	8.1	6.4	79	0.56	6.9	

He was then given 10 gm. of taurine by mouth and similar determinations were carried out for this day as well as for several succeeding days. The results are given in Table I. It is at once evident that ingestion of taurine has resulted in an increase of both neutral sulfur and α-amino nitrogen, in amounts which correspond to about 63 per cent of the ingested taurine. There is no increase in the slowly reacting amines, indicating that no appreciable amount of taurine has combined with urea to form taurocarbamic acid. On the basis of increase in total nitrogen excreted, the amount of taurine eliminated is greater than that calculated from the sulfur or α-amino nitrogen. Since there is a greater variability in the total nitrogen figures the latter method of calculation seems the more accurate.

Several other experiments were carried out to determine the effect, if any, of the ingestion of taurine on the excretion of urea and ammonia in urine (Table II). If urea and taurine combined *in vivo* and the reaction follows the same course as *in vitro*



a decrease in urea and a corresponding increase in ammonia nitrogen should be expected. Within the normal variability the amounts of urea and ammonia excreted are not affected by the administration of taurine. These figures support those of the previous experiment and point to the conclusion that taurocarbamic acid is not a metabolic end-product when taurine is ingested.

SUMMARY.

1. Determinations of neutral sulfur and amino-acid nitrogen in the urine of an individual given 10 gm. of taurine by mouth show a close agreement in the increase of α-amino nitrogen and neutral sulfur over the control period, indicating that taurine is not eliminated as taurocarbamic acid.

2. Administration of taurine in large doses does not result in any marked changes in the excretion of urea and ammonia.

SOME PROTEINS FROM THE GEORGIA VELVET BEAN, STIZOLOBIUM DEERINGIANUM.

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(Received for publication, March 8, 1920.)

The Georgia velvet bean is a sport of the Florida velvet bean, *Stizolobium deeringianum*, from which it differs principally in having the shortest season of all the cultivated *Stizolobia*, so that it can be successfully grown in the extreme northern part of the cotton belt. It is used chiefly as cattle food and in 1917 its production had already increased to such an extent as to exceed that of any other plant of the group. The beans themselves are of a mottled color, dark brown and white. They are spherical to ovoid in shape, slightly flattened, about $\frac{3}{8}$ inch in greatest diameter, and extremely hard and tough.

An examination of the proteins of the closely related Chinese velvet bean, *Stizolobium niveum*, has already been published by this laboratory.[1] The increasing importance of the Georgia strain, however, seemed to make it desirable that the latter also should be made the subject of a special study.[2] Considerable differences in the nature of the protein content of the two seeds have been brought out, as will be seen if comparison is made of this with the preceding paper.

The Georgia velvet bean contains about 23.6 per cent of protein ($N \times 6.25$). 3 per cent sodium chloride solutions extract a maximum of about 15 per cent of protein from the finely ground seed; and a yield of 13 per cent of mixed proteins is obtainable by

[1] Johns, C. O., and Finks, A. J., *J. Biol. Chem.*, 1918, xxxiv, 429.

[2] For information concerning the development, culture, and use of velvet beans we are indebted to Dr. C. V. Piper of the Bureau of Plant Industry of the Department of Agriculture. The beans used in this investigation were furnished by the Bureau of Plant Industry.

coagulation from the slightly acidified extract at the boiling temperature.

From the sodium chloride extracts we have separated three types of protein: a globulin preparation, referred to in this paper as the α-globulin, which is precipitated from the extract by 0.4 of saturation with ammonium sulfate, coagulates at 70–78°C., and contains 0.90 per cent of sulfur; a second type of globulin preparation, which we have designated the β-globulin, requiring 0.6 to 0.7 of saturation with ammonium sulfate for precipitation, coagulating at 90–100°C., and containing but 0.45 per cent of sulfur; and an albumin type, obtained by coagulation from aqueous extracts previously freed from globulin by prolonged dialysis. The

TABLE I.

Percentages of the Basic Amino-Acids in the Proteins from the Georgia Velvet Bean.

Amino-acid.	α-Globulin.		β-Globulin.		Albumin.
	I	II	I	II	I
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Cystine	1.03	1.04	0.91	0.87	1.92
Arginine	7.25	7.14	8.09	8.28	6.13
Histidine	1.34	1.14	3.32	3.43	0.82
Lysine	8.20	8.44	8.61	8.42	8.20

globulins are thus precipitated, and are removed by decantation and filtration, while the albumin remains in solution. This coagulates at 54–62°C., and contains about 1.00 per cent of sulfur.

Particularly striking is the behavior of these preparations in the qualitative test for *tryptophane* by Hopkins and Cole's reaction. The α-globulin shows strongly the color reaction characteristic of tryptophane; but the β-globulin gives no trace of the reaction, even after long standing. The albumin preparations give the test at once, and after several hours standing the color becomes very intense.

The percentages of the basic amino-acids in these proteins, as determined by Van Slyke's method,[3] are given in Table I.

[3] Van Slyke, D. D., *J. Biol. Chem.*, 1911-12, x, 15.

EXPERIMENTAL.

Preliminary.

Extraction Experiments.—The whole beans were crushed in a power-driven meat chopper and ground to a fine meal in a pulverizing mill. Extractions were then made to ascertain the solvent best suited for the extraction of the proteins. 10 gm. samples of the meal were thoroughly stirred with 200 cc. portions of the solvent. The mixtures were allowed to stand for 24 hours at room temperature in order to secure as complete an extraction as possible. The supernatant liquid was then filtered off through small folded filters, and nitrogen determined in 20 cc. aliquots. The results (Table II) indicate 3 per cent salt solution as the most effective of the solvents tried. This solution was used for the extraction of the globulin preparations described in this paper.

TABLE II.

Preliminary Extraction Experiments.

Solvent (NaCl).	Nitrogen extracted from meal.*	Protein extracted from meal.*
per cent	*per cent*	*per cent* ($N \times 6.25$)
10	1.75	10.94
7	2.04	12.73
5	2.30	14.41
3	2.42	15.09
1	2.23	13.95
0.1	1.66	10.36

* Computated as percentage of total dry substance.

Precipitation with Ammonium Sulfate.—To determine the behavior of the proteins in the saline extract toward ammonium sulfate, 10 cc. portions of the extract were made successively from 0.1 to 0.9 saturated with the precipitant, filtering after each addition through a small folded filter moistened with an ammonium sulfate solution of the same concentration as that of the test solution. These tests indicated the presence of but *one* globulin fraction, that precipitated by 0.4 of saturation with ammonium sulfate; and a number of preparations were made. It was later observed, however, in working with large volumes of extract (5 or 6 liters), that a small further precipitate was produced by increasing the concentration of ammonium sulfate to 0.6 of saturation. Separations of the two globulin fractions were then made as described in the following section.

The α- and β-Globulins.

Extraction.—2 or 3 kilos of meal were thoroughly stirred with five volumes of a 3 per cent distilled water solution of pure sodium chloride. The extraction was allowed to continue for 24 hours at about 2°C. Filter paper scrap was then added in sufficient quantity to produce a pulp of a consistency suitable for pressing, the pulp placed in muslin bags, and the liquid pressed out in a powerful press. The resulting turbid extract was filtered by suction through thick mats of filter paper pulp on Buchner funnels and the filters were washed once with a small volume of 3 per cent salt solution.

Preparation of the α-Globulin.—The filtered extract was measured and made 0.4 saturated with ammonium sulfate. The finely powdered precipitant was added slowly and with vigorous stirring, each portion being completely dissolved before more was added. The voluminous precipitate became flocculent and settled in about ½ hour. It was filtered off on a folded filter, washed from the filter with 0.4 saturated ammonium sulfate solution, again filtered off, and finally redissolved with distilled water, the ammonium sulfate which remained in the paste supplying in this case the neutral salt necessary for the solution of the globulin. This solution, after filtering clear, was dialyzed 10 or 12 days in parchment paper bags against running cold water. The precipitate thus formed was washed free from remaining traces of chlorides with distilled water, dehydrated by suspension for 24 hours in absolute alcohol, freed from alcohol by washing on a Buchner funnel with absolute ether, and dried by gradually heating to 110°C. in a vacuum oven. Preparations made in this manner constituted the α-globulin. The yield is from 2.75 to 3.00 per cent of the meal extracted.

Preparation of the β-Globulin.—The filtrate and washings from the original precipitate of α-globulin were measured and made 0.5 saturated with ammonium sulfate and the extremely small precipitate was removed. From the filtrate the β-globulin was thrown out by adding ammonium sulfate up to 0.7 of saturation. Most of this fraction is thrown down at 0.6 of saturation with the precipitant, and separation is complete at less than 0.7 of saturation. The purification and drying of this material were carried out exactly as described for the α-globulin. The yield is about 1.25 per cent of the meal extracted.

Physical Properties.—The precipitation limits of the globulins with ammonium sulfate have been noted in the preceding paragraphs. The coagulation temperatures were determined by redissolving small portions of the dialysis precipitates in 3 per cent salt solution, adding 0.5 per cent of acetic acid, and heating at the rate of about 0.5°C. per minute until coagulation began and then very slowly until the precipitate was flocculent and the supernatant liquid clear. The apparatus used was similar to that employed in the determination of melting points, except that a

test-tube containing the solution under examination with a thermometer immersed therein was substituted for the thermometer with attached melting point tube. Under these conditions the α-globulin shows cloudiness at 70°C., a flocculent precipitate at 74–75°C., and apparently complete coagulation with the supernatant liquid clear at 78°C. The β-globulin clouds at about 90°C., shows a flocculent appearance at 97°C., and the cloudiness of the supernatant liquid disappears but slowly at the boiling temperature.

No evidence of crystalline form could be observed in the dialysis precipitates of either of the globulins. The appearance of the α-globulin under the microscope was that of extremely minute, amorphous, translucent particles; the β-globulin was in all respects similar, except that the particles were considerably coarser. The finished preparations are pale gray, dusty powders, light, and decidedly hygroscopic; exposed to the air, they absorb from 6 to 10 per cent of moisture.

Qualitative Examination.—The ordinary protein reactions are shown by both globulins, with the exception of the reaction of Hopkins and Cole, which depends upon the presence of *tryptophane*. The color characteristic of this amino-acid develops immediately, and strongly, when the test is applied to the α-globulin, but careful and repeated testing of all our preparations of the β-globulin failed to show any trace of the reaction.[4] This is particularly interesting inasmuch as no vegetable globulin has hitherto been shown to lack tryptophane. The *prolamin*, zein, from *Zea mays* is the only vegetable protein which heretofore has been shown to be entirely without this amino-acid.

Molisch's test indicates the presence of but traces of carbohydrate in the α-preparations, since the color given is very faint. The β-preparations, with one exception, did not show the reaction at all; in the one case in which the test was positive the color was barely detectable. The faint colors produced in this very sensitive test would seem to indicate that the traces found are impurities merely, probably filter paper fibers.

Phosphorus was not found in either protein.

[4] Osborne and Mendel have been kind enough to test this β-globulin for us, and have confirmed our results.

TABLE III.

α-Globulin.

	Preparation I.				Preparation II.			
	I	II	Average.	Ash-free.	I	II	Average.	Ash-free.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Carbon.....	52.49	52.68	52.59	53.03	52.80	52.89	52.85	52.97
Hydrogen...	6.78	6.69	6.74	6.80	6.77	6.81	6.79	6.81
Nitrogen....	16.58	16.62	16.60	16.74	16.72	16.72	16.72	16.76
Sulfur.....	0.92	0.86	0.89	0.90	0.91		0.91	0.91
Oxygen*....				22.53				22.53
Ash.....	0.79		0.79		0.24		0.24	
	Preparation III.				Preparation IV.			
Carbon.....	53.09	53.30	53.20	53.36	52.39	52.45	52.42	52.97
Hydrogen...	6.79	6.92	6.86	6.88	6.71	6.72	6.71	6.79
Nitrogen....	16.66	16.69	16.68	16.73	16.20	16.28	16.24	16.41
Sulfur.....	0.93		0.93	0.93	0.88		0.88	0.89
Oxygen*....				22.10				22.94
Ash.....	0.32		0.32		0.93		0.93	

* By difference.

TABLE IV.

β-Globulin.

	Preparation I.				Preparation II.			
	I	II	Average.	Ash-free.	I	II	Average.	Ash-free.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Carbon.....					52.85	52.87	52.86	53.16
Hydrogen...					6.77	6.76	6.77	6.80
Nitrogen....					17.09	16.93	17.01	17.11
Sulfur.....	0.44	0.45	0.45	0.45	0.45		0.45	0.46
Oxygen*....								22.47
Ash.....	0.63		0.63		0.55		0.55	
	Preparation III.				Preparation IV.			
Carbon.....	53.29	53.28	53.29	53.34	52.66	52.69	52.68	52.99
Hydrogen...	6.71	6.78	6.75	6.76	6.75	6.79	6.77	6.81
Nitrogen....	17.18	17.07	17.13	17.17	17.54	17.35	17.45	17.55
Sulfur.....	0.45		0.45	0.46	0.44		0.44	0.45
Oxygen*....				22.26				22.20
Ash.....	0.11		0.11		0.58		0.58	

* By difference.

Elementary Composition.—On account of their hygroscopic properties which render the accurate weighing of dry samples impossible, all preparations were exposed in thin layers until equilibrium with the moisture of the air was reached. Moisture and

TABLE V.

*Distribution of Nitrogen in the α-Globulin as Determined by Van Slyke's Method.**

Sample I, moisture- and ash-free, 3.0212 gm. protein, 0.5057 gm. nitrogen.†
" II, " " " " 2.9788 " " 0.4984 " "

	I	II	I	II	Average.
	gm.	*gm.*	*per cent*	*per cent*	*per cent*
Amide N	0.0457	0.0445	9.04	8.93	8.99
Humin N adsorbed by lime	0.0077	0.0064	1.53	1.30	1.42
" N in amyl alcohol extract	0.0002	0.0015	0.04	0.30	0.17
Cystine N	0.0037	0.0036	0.73	0.72	0.73
Arginine N	0.0696	0.0695	13.75	13.95	13.85
Histidine N	0.0093	0.0108	1.85	2.17	2.01
Lysine N	0.0490	0.0469	9.69	9.42	9.56
Amino N of filtrate	0.2994	0.2952	59.20	59.23	59.22
Non-amino N of filtrate	0.0218	0.0210	4.32	4.22	4.27
Total nitrogen recovered	0.5064	0.4994	100.15	100.24	100.22

* Nitrogen figures corrected for the solubilities of the bases.
† Nitrogen content of protein, 16.73 per cent.

TABLE VI.

Basic Amino-Acids in the α-Globulin.

Amino-acid.	I	II	Average.
	per cent	*per cent*	*per cent*
Cystine	1.03	1.04	1.04
Arginine	7.25	7.14	7.20
Histidine	1.34	1.14	1.24
Lysine	8.20	8.44	8.32

ash were then determined, and all analyses calculated on the moisture-free basis. The results are given in Tables III and IV.

Distribution of Nitrogen in the Globulins as Determined by Van Slyke's Method.—Duplicate samples, about 3 gm., of each of the

globulins were hydrolyzed by boiling for 24 hours with 100 cc. of 20 per cent hydrochloric acid. The phosphotungstates of the bases were decomposed by the amyl alcohol-ether method.[5] The results are given in Tables V, VI, VII, and VIII.

TABLE VII.

*Distribution of Nitrogen in the β-Globulin as Determined by Van Slyke's Method.**

Sample I, moisture- and ash-free, 2.8713 gm. protein, 0.4918 gm. nitrogen.†
" II, " " " 2.8708 " " 0.4917 " "

	I	II	I	II	Average.
	gm.	*gm.*	*per cent*	*per cent*	*per cent*
Amide N	0.0532	0.0536	10.81	10.89	10.85
Humin N adsorbed by lime	0.0070	0.0074	1.41	1.50	1.46
" N in amyl alcohol	0.0002	0.0002	0.04	0.04	0.04
Cystine N	0.0031	0.0029	0.63	0.59	0.61
Arginine N	0.0748	0.0766	15.21	15.57	15.39
Histidine N	0.0259	0.0267	5.26	5.43	5.34
Lysine N	0.0475	0.0464	9.65	9.44	9.55
Amino N of filtrate	0.2580	0.2572	52.46	52.30	52.38
Non-amino N of filtrate	0.0229	0.0192	4.65	3.90	4.28
Total nitrogen recovered	0.4926	0.4902	100.12	99.66	99.89

* Nitrogen figures corrected for the solubilities of the bases.
† Nitrogen content of protein, 17.13 per cent.

TABLE VIII.

Basic Amino-Acids in the β-Globulin.

Amino-acid.	I	II	Average.
	per cent	*per cent*	*per cent*
Cystine	0.91	0.87	0.89
Arginine	8.09	8.28	8.19
Histidine	3.32	3.43	3.38
Lysine	8.61	8.42	8.52

The Albumin.

Preparation.—1 or 2 kilos of meal were stirred with five volumes of distilled water and the extraction was allowed to continue over night at about 2°C. The mixture was then prepared and pressed and the extract filtered,

[5] Van Slyke, D. D., *J. Biol. Chem.*, 1915, xxii, 281.

exactly as in the preparation of the globulins. The extract was dialyzed directly, without previous treatment with ammonium sulfate, for 10 or 12 days and the precipitated globulin removed by decantation and filtration. The clear filtrate was dialyzed again for several days, the precipitate removed, and the dialysis resumed. This process was continued till the precipitation of the globulins appeared to be complete. The albumin was obtained from the filtered dialysis liquors by coagulation at a temperature which was not allowed to exceed 65°C. (the coagulation range is from 54–62°C.); this coagulum, after washing with warm distilled water until the last washings were free from chlorides, was dried by means of absolute alcohol and ether with final heating to 110°C. *in vacuo*, as was done in the case of the globulin preparations. The yield is 0.6 to 0.75 per cent.

Physical Properties.—The albumin coagulates from its slightly acidified solution within the range 54–62°C. The finished preparations are very similar in appearance to those of the globulins, though somewhat lighter in color.

Qualitative Examination.—The ordinary protein reactions are given by this material. Hopkins and Cole's color reaction for tryptophane develops slowly but becomes very strongly positive on standing. Traces of carbohydrate are present; probably the feebly positive test is due to minute quantities of filter paper fibers.

Elementary Composition.—The same procedure was adopted as in the analysis of the globulins, the results being calculated on the moisture-free basis. The albumin preparations are sharply differentiated from those of either of the globulins by their higher sulfur and lower nitrogen content. The analysis is summarized in Table IX.

Nitrogen Distribution in the Albumin as Determined by the Van Slyke Method.—Owing to the extremely small yield of this protein, material for duplicate determinations was not obtained. 2.0079 gm. of the albumin, equivalent to 1.8327 gm. of moisture- and ash-free protein, were hydrolyzed by boiling with 100 cc. of 20 per cent hydrochloric acid for 24 hours. Analysis of the hydrolysate was then made according to Van Slyke's directions, using the amyl alcohol-ether method[5] for the decomposition of the precipitate of the basic amino-acids. The calculated nitrogen of the sample is 0.2931 gm.; recovered, 0.2933 gm. The figures obtained are given in Tables X and XI.

TABLE IX.

Albumin.

	Preparation I.				Preparation II.			
	I	II	Average.	Ash-free.	I	II	Average.	Ash-free.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Carbon	52.61	52.76	52.69	53.36	49.51	49.49	49.50	53.43
Hydrogen	6.72	6.67	6.70	6.80	6.27	6.20	6.24	6.73
Nitrogen	15.67	15.81	15.74	15.96	15.05		15.05	15.76
Sulfur	0.99	1.01	1.00	1.02	0.97		0.97	1.00
Oxygen*				22.86				23.08
Ash	1.23		1.23		3.75		3.75	

* By difference.

TABLE X.

*Distribution of Nitrogen in the Albumin as Determined by Van Slyke's Method.**

Sample, ash- and moisture-free, 1.8327 gm. protein, 0.2931 gm. nitrogen.†

	gm.	*per cent*
Amide N	0.0266	9.08
Humin N adsorbed by lime	0.0053	1.80
" N in amyl alcohol extract	0.0000	0.00
Cystine N	0.0041	1.41
Arginine N	0.0362	12.35
Histidine N	0.0041	1.41
Lysine N	0.0289	9.85
Amino N of the filtrate	0.1881	64.25
Non-amino N of the filtrate	0.0010	0.34
Total nitrogen regained	0.2943	100.49

* Nitrogen figures corrected for the solubilities of the bases.

† Nitrogen content of protein, 15.99 per cent.

TABLE XI.

Basic Amino-Acids in the Albumin.

	per cent
Cystine	1.92
Arginine	6.13
Histidine	0.82
Lysine	8.20

SUMMARY.

The Georgia velvet bean contains 23.6 per cent of protein (N×6.25). Experiments with sodium chloride in various concentrations indicate a 3 per cent aqueous solution as the most effective solvent. This solution extracts about 15 per cent of protein.

From the 3 per cent sodium chloride extracts a yield of about 13 per cent of mixed proteins may be secured by coagulation with heat. By the fractional precipitation of the extract with ammonium sulfate, however, and by subsequent purification of the fractions as described in this paper two globulins, designated as the α- and β-globulins, may be separated, in yields of about 3 and 1.25 per cent, respectively; and an albumin is found in extracts which have been freed from globulin by repeated dialysis. The latter protein may be coagulated in yields of about 0.75 per cent by the prolonged heating of its slightly acidified solution at 65°C.

Analyses of the three proteins show marked differences in the nitrogen and sulfur content, and in the distribution of nitrogen as determined by Van Slyke's method.

Qualitative examination indicates that the β-globulin contains no tryptophane, an observation of particular interest in view of the fact that this amino-acid has been found to be present in all seed globulins heretofore tested. The α-globulin and the albumin from the Georgia velvet bean both contain tryptophane.

EXPERIMENTAL STUDIES ON GROWTH.*

XV. ON THE GROWTH OF RELATIVELY LONG LIVED COMPARED WITH THAT OF RELATIVELY SHORT LIVED ANIMALS.

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Experimental Results.

In previous articles of this series[1] experiments have been described in which the growth of white mice fed upon a varied and abundant diet, to which specified unusual additions had been made, was compared with the growth of similar animals fed upon the same diet but without the unusual additions. Since these experiments and observations were continued without interruption from shortly after the birth of the animals until the occurrence of death from natural causes, they offer the opportunity of instituting a highly important comparison, which has not heretofore been attempted; namely, the comparison at stated brief intervals of the growth in weight of animals which survive to an age in excess of the average duration of life with that of animals in which death antedates the average duration of life. Since individual records of the weights of all the animals were kept throughout, and deaths from essentially accidental causes, such as epidemic infections, were reduced by our technique to a minimum[2] this comparison could be made by simply sorting the animals of each experimental group into two subgroups, the one,

* The expenses of this research were in part defrayed by a grant from the Special Medical Research Fund of the University of Toronto.

[1] Robertson, T. B., and Ray, L. A., *J. Biol. Chem.*, 1916, xxiv, 347; 1919, xxxvii, 377, 393, 427, 455. Robertson, T. B., *J. Biol. Chem.*, 1916, xxiv, 363, 385, 397; xxv, 635, 647.

[2] Robertson, T. B., and Ray, L. A., *J. Biol. Chem.*, 1916, xxiv, 347; 1919, xxxvii, 443.

the short lived group, of animals dying of causes other than accidental injury or epidemic infection before the average duration of life for the group under consideration, and the other, the long lived group, of animals dying subsequently to the average duration of life. The weights of each of the subgroups are averaged week by week until 7 months of age and fortnightly thereafter. The results of this comparison are enumerated in Tables I to XI.[3]

An inspection of these tables reveals the following facts:

1. In nine out of the eleven different experimental groups the long lived animals display evidence of superior growth velocity to the short lived animals at some time prior to the 30th week of age. Exceptions are afforded by the normal males and the lecithin-fed males.

2. At later ages there is a decided tendency for the curve of growth of the short lived animals to approach or cut across the curve which represents the growth of the long lived animals. This is due to the tendency of the short lived group to acquire a late accretion of tissue. This gain is, however, unstable and usually of comparatively brief duration, the curve dropping to the level of the long lived group or below it as the termination of life is approached.

3. In eight out of the eleven different experimental groups the long lived animals are less variable than the short lived animals. Exceptions are afforded by the normal females and both groups of tethelin-fed females.

4. Difference between the two groups is, however, most markedly displayed in their mode of reaction to external disturbing factors. All growth curves reveal minor fluctuations, accompanied by transient increases in variability, which are presumably attributable to unidentifiable fluctuations of the environment. The short lived animals are, however, much less stable than the long lived; their average weight is subject to more numerous and violent fluctuations and their variability increases abnormally towards the latter part of the curve. A disturbance of the environment may be reflected by the long lived group in enhanced

[3] For details as to dosages of the dietary additions, number of animals employed, experimental procedure, *etc.* consult the previous articles of this series.

variability without concurrent alteration of weight, while the short lived animals simultaneously display relatively rapid gains or somewhat slower losses.[4]

Summarizing these results we may state therefore that as a general rule, when long and short lived animals which have been subjected to the same experimental treatment are compared, the long lived animals form a group which in early life grow more rapidly and at the same time are less variable than the short lived animals. In later life the short lived animals often grow much more rapidly than the long lived animals, but this accretion of tissue is relatively unstable.

So far we have restricted our comparison to the long and short lived animals respectively of the same experimental group. When we extend the comparison further and compare the long lived animals of one experimental group with those of another we find that there is no characteristic type to which the growth curve of long lived or short lived animals tends to conform. A glance at Figs. 1 and 2, in which all the various growth curves for males and females respectively are plotted to the same scale, shows that there is no tendency for the curves of the long lived animals to lie together or to be appreciably separated from the group of curves representing the growth of the short lived animals. On the contrary the curves are interwoven with one another. There is in fact much more tendency for a given curve to resemble its dietary similar than its longevity similar. Thus in both diagrams the curves for the long lived and short lived pituitary-fed animals tend to accompany one another, both as to form and position, and the same is true for the lecithin and tethelin curves. In other words the various dietaries employed in these experiments had a much more decided effect upon the time relations of growth than they had upon the longevity of the animals to which they were administered. This confirms the conclusion formulated in a previous article;[5] namely, that the substances administered, with the exception of tethelin, had no decided effect upon the life duration of the animals.

[4] This is true long before the numbers of the short lived animals have been decreased by deaths.

[5] Robertson, T. B., and Ray, L. A., *J. Biol. Chem.*, 1919, xxxvii, 427.

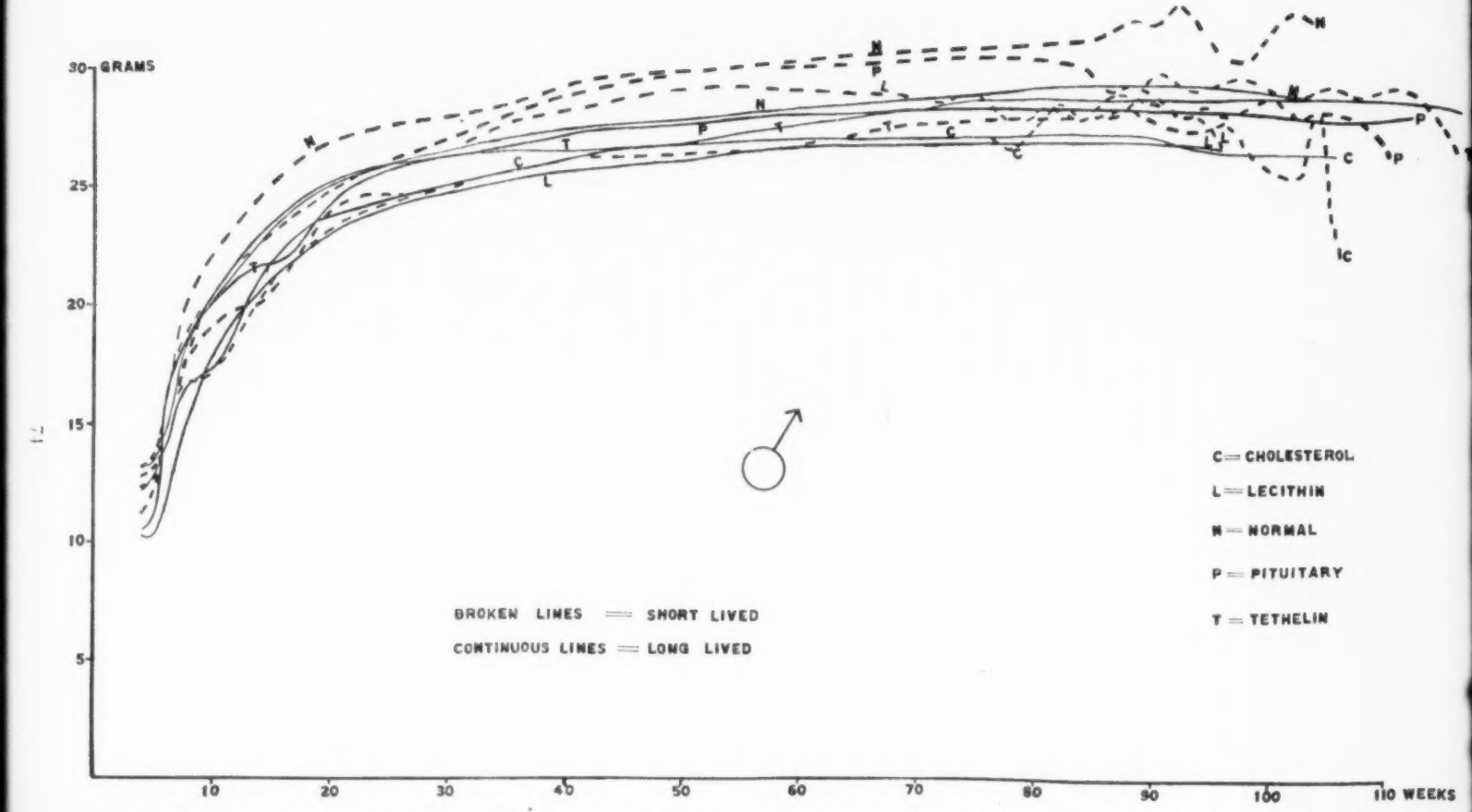

FIG. 1. Comparison of the growth curves of relatively long lived and relatively short lived males. There is no single type of curve pertaining to longevity.

FIG. 1. Comparison of the growth curves of relatively long lived and relatively short lived males. There is no single type of curve pertaining to longevity.

FIG. 2. Comparison of the growth curves of relatively long lived and relatively short lived females. There is no single type of curve pertaining to longevity. The supernormal growth of the long lived animals which received a brief preadolescent administration of tethelin is clearly displayed.

A correlation may nevertheless be traced between the time relations of growth and the longevity of the animals which display them. It is masked, but not concealed, by regulatory internal factors which tend to maintain the normal standard of longevity. Thus we have seen that in almost every group the long lived animals grow initially, or at an early stage, more rapidly than the short lived animals and that in comparatively late life the short lived animals display an unstable increase in weight. This late accretion of tissue would appear to be exceptionally unfavorable to longevity. Thus the longest lived animals in the series, those fed upon tethelin throughout the period of their lives (Table V) or intermittently (Table VI), do not display it at all, the curve of growth from the 40th to the 100th week being almost a straight line, and the average weight during this period increases very slightly.[6] The opposite extreme is afforded by those animals which have received tethelin for only a brief period antedating sexual maturity (4th to 12th weeks). As we have previously pointed out,[7] these animals display an extraordinary degree of late overgrowth, to which the curves marked TB in Fig. 2 bear striking witness.[8] These animals not only displayed no increased duration of life but were actually less viable than normal females, the short lived group especially being the least viable of all the experimental groups.

Increased viability is accompanied by a tendency for the short lived group to approach the long lived group in life duration, and conversely a brief average duration of life is accompanied by an enhanced difference between the viability of the two groups. This is shown by Table XII in which the different experimental groups are arranged in the descending order of their mean duration of life.

It is evident that the dispersion of deaths, as measured by the ratio of the difference between the life durations of the long and short lived animals to the mean life duration of the group, is least in the longest lived groups and progressively increases as the viability decreases. The increased scattering of deaths with

[6] Robertson, T. B., and Ray, L. A., *J. Biol. Chem.*, 1919, xxxvii, 393.

[7] Robertson, T. B., and Ray, L. A., *J. Biol. Chem.*, 1919, xxxvii, 455.

[8] No measurements of the weights of these animals were made between the 30th and the 70th weeks.

decrease of viability is due to the fact that while the viability of the long lived groups is affected it is not so greatly affected by external or dietary circumstances as the viability of the short lived group. Thus in passing downwards among the males from an average life duration of 866 days to one of only 731 days, the life duration of the long lived group diminishes by 36 days, while that of the short lived group diminishes by 199 days. The same tendency is exhibited by the females (compare the tethelin continuous administration group with the tethelin brief administration) but not so conspicuously.

Discussion and Interpretation of the Results.

In attempting the comparison of the growth of more viable as contrasted with that of less viable animals we are constrained to adopt some standard of viability which must of necessity be more or less arbitrary. The standard herein adopted is the average duration of life of any given experimental group and animals are grouped as long or short lived in comparison with this standard. It is the only practicable standard, and yet it is far from satisfactory, for a certain proportion of the short lived animals, that is, of the animals dying before the average life duration of the group, must necessarily die of causes which are essentially accidental although not clearly recognizable as such. Thus an animal which might otherwise have lived to the most advanced age afforded by the species develops a sarcoma or an infection and dies prematurely. We cannot definitely assert that in these cases, as in carcinoma, the lesion is consequent upon age or necessarily correlated with age. We can safely aver that senescence of the tissues must lay them open to disease, but we cannot conversely assert that disease proves senescence. But if these animals, dying of causes unconnected with the age of their tissues, were recognizable and excluded from the comparison, the average duration of life would thereby be advanced to a point which we have no means of ascertaining, and the more viable group would be correspondingly restricted in numbers.

If the true average duration of life of any group of animals, in so far as this is decided by tissue age alone, were ascertainable, therefore, the proportion of animals herein regarded as most

viable would be reduced by exclusion of less viable individuals, while certain individuals would be excluded altogether from the less viable group or even included, despite their early death, among the members of the more viable group. All that the foregoing data can be expected to reveal, therefore, is a more or less pronounced *tendency* of the two groups to differ, a tendency which must be masked to a certain extent by the imperfect means of distinction employed.

This tendency is very plainly evinced and, as we have seen, it consists in initial relative supernormality of growth of the long lived group, accompanied by a subnormality of variability. The only exception to both these rules is afforded by the group of "normal males." The origin of this single exception among eleven different experimental groups may not improbably lie in the elements of uncertainty we have enumerated.

The association of superior growth with inferior variability is very remarkable, because it is precisely the opposite of what we find in comparisons of groups differentiated by any other criterion than that of longevity.[9] If different experimental groups or ages of animals or of man are compared, it is always found that the most rapidly growing and, age for age, the largest animals, are also the most variable.

The converse association, namely that of retarded growth with high variability, is characteristic of the effects of a highly unfavorable environment.[10] We may therefore infer that the long lived animals, displaying as they do the reverse tendency, are unusually immune to deleterious factors in their environment. They are a relatively stable group. Internal regulatory processes tend promptly and accurately to restore disturbed equilibria, and, as we have seen, this relative stability is also evidenced by the comparative rarity of irregular fluctuations of weight.

The relative internal stability of the long lived groups of animals is especially displayed by the events which occur at the period of life which corresponds with the average duration of life of the short lived groups. Factors which are at present unknown

[9] Robertson, T. B., *J. Biol. Chem.*, 1916, xxiv, 363; *Am. J. Physiol.*, 1916, xli, 535. Thompson, D'A. W., On growth and form, New York, 1917.

[10] Robertson, T. B., *J. Biol. Chem.*, 1916, xxv, 635, 647; *Am. J. Physiol.*, 1916, xli, 547.

disturb at this point the stability of the animals, and this disturbance is reflected in increased variability of both the long lived and the short lived groups. But in the long lived groups this is accompanied by little or no disturbance of weight, while in the short lived group it results in loss, or in sharp gain followed by loss of weight.

The long lived animals therefore represent a relatively stable group, highly resistant to external disturbing factors, displaying subnormal variability and a more or less marked, but not invariable, tendency to early overgrowth and relative paucity of tissue accretion in late life.

The short lived animals, on the contrary, are relatively unstable, sensitive to external disturbing factors, supernormally variable, and as a rule, but not invariably, display relatively deficient early growth and a tendency to rapid accretion of tissue in late life.

Before endeavoring to interpret these results we must revert to a peculiarity which distinguishes the action of agents known to affect the rate of growth of tissues and the longevity of animals to which they are administered. It has been shown in a number of previous communications that cholesterol[11] and tethelin[12] accelerate the growth and reproduction of unicellular organisms (*Paramecia*) and of epithelial tissues, especially carcinoma. Tethelin also accelerates the recovery of weight lost during inanition. Yet these agents which accelerate the development or multiplication of the tissue elements which we have enumerated nevertheless *retard* the growth in weight of animals to which they are administered by mouth.[13] We can only infer that there must be, in the totality of tissues which constitute the higher Metazoa, certain tissues, and those the relatively more bulky, which are retarded in their development, directly or indirectly, by these agents.

On considering the nature of the tissues which are positively known to be accelerated in their development by cholesterol

[11] Robertson, T. B., and Burnett, T. C., *J. Exp. Med.*, 1913, xvii, 344. Browder, A., *Univ. of California Pub. Physiol.*, 1915, v, 1. Sweet, J. E., Corson-White, E. P., and Saxon, G. J., *J. Biol. Chem.*, 1915, xxi, 309.

[12] Robertson, T. B., *J. Am. Med. Assn.*, 1916, lxvi, 1009. Robertson, T. B., and Burnett, T. C., *J. Exp. Med.*, 1915, xxi, 280; 1916, xxiii, 631.

[13] Robertson, T. B., *J. Biol. Chem.*, 1916, xxiv, 397; 1919, xxxvii, 393. Robertson, T. B., and Delprat, M., *J. Biol. Chem.*, 1917, xxxi, 567.

and by tethelin the first point of resemblance which presents itself lies in the fact that they are all preeminently cellular or parenchymatous tissues or tissue elements. Now there exists in the body of all the higher Metazoa a considerable mass of tissue, usually the relatively more bulky, which is not primarily cellular or parenchymatous in character, but consists of fibrils or other specialized elements having, on the whole, a primarily structural rather than a primarily vital significance to the animal. They are produced from cell elements and ultimately derive from them the materials for their nutrition, maintenance, and repair; they are dependent tissues and may to a large extent be regarded as ultimately parasitic upon the parenchymatous elements.[14]

These primarily structural or sclerous elements of the body are constituted for the most part of substances which are characteristic of the particular type of tissue which contains them, substances which contribute to its peculiar physical properties, its rigidity, elasticity, tensile strength, or other essential quality. This is especially true of the protein constituents of these tissues and it is precisely from tissues of this character that we obtain aberrant or "incomplete" proteins, deficient in this or that amino-acid and characterized by their abnormal content of this or that other amino-acid. To produce such protein of abnormal composition requires the destruction and resorting of the components of several or perhaps many molecules of the more "typical" proteins which occur in parenchymatous tissue. The connective tissues are therefore relatively expensive to support and dependent as they are, directly or indirectly, upon parenchymatous tissues for their maintenance they must cast collectively a heavy load upon the metabolism and maintenance of the most vital tissues in the body.

In any system of parallel chemical reactions which consume the same substrate and are proceeding simultaneously in a mixture, the extent to which each reaction will occur is proportionate to its velocity.[15] The most rapid reaction will predominate over the rest and appropriate the greater share of the sub-

[14] Adami, J. G., Principles of pathology, Philadelphia and New York, 1908, i, 125.

[15] Mellor, J. W., Chemical statics and dynamics, London, New York, and Bombay, 1904, 70.

strate. In the tissues of the Metazoa, the substrates are the foodstuffs which are present in the circulating fluids which bathe the tissue elements and the reactions of synthesis which underlie growth, repair, and maintenance share among them in a definite proportion the various nutritive materials which are available. If, therefore, any circumstance, such as the introduction of a catalyzer, specifically accelerates the syntheses occurring in one type of tissue to the exclusion of others, this type of tissue will acquire an advantage in the appropriation of nutriment and the other tissues will in like proportion be retarded in their development, maintenance, or repair.

We have seen that the cellular tissues are definitely accelerated in their growth and repair by cholesterol and tethelin, while the growth of animals as a whole is retarded by these agents. It seems necessary to infer that certain tissues other than the distinctively cellular elements are retarded in their development by cholesterol and tethelin. The only considerable group to which we can attribute this retardation of growth in weight is the group of sclerous or dependent tissues.

An increase in connective tissue elements is commented upon by all observers[16] as the most distinctive characteristic of old age, and when we reflect that these tissues are ultimately dependent upon parenchyma for their support and that they are exceptionally expensive to maintain we can readily comprehend how an ever increasing accumulation of tissues of this character may place an excessive and finally unbearable load upon the metabolism of the cellular tissues. On the other hand a very high proportion of cellular elements is characteristic of embryonic tissue.

If this view is correct, then the problem of the prolongation of life is essentially that of deferring the accretion of connective tissues which occurs in the later life of all the higher Metazoa. If we have correctly interpreted the influence of tethelin upon the growth of animals as consisting in acceleration of the anabolism of the cellular elements to the detriment of the connective tissue elements, then the decided prolongation of life which adminis-

[16] Saundby, R., Old age; its care and treatment in health and disease, London, 1913, Chapter 2. Metchnikoff, E., The nature of man, New York and London, 1913; The prolongation of life, New York and London, 1910.

tration of this agent brings about[5] is readily comprehended. Cholesterol, which in like manner but in greater dosage accelerates the growth of cellular tissues, does not bring about a like prolongation of life, but for this there is a sufficient reason; namely, that deposits of this insoluble material are formed in various organs, to their detriment, when it is administered in excess.[17]

If, however, the stimulus supplied by tethelin to the anabolism of cellular elements is removed, at any rate if it is removed before sexual maturity is attained, the normal proportionality between cellular and sclerous elements tends rapidly to be restored; the overgrowth which has actually occurred but has been masked by the retarded development of sclerous tissues is now rendered manifest, and gigantic animals may be produced. In illustration Fig. 3 shows a normal female of average normal weight at 500 days in comparison with a female of the average weight of twenty-four animals which had received 4 mg. of tethelin daily from the 4th until the 12th week after birth, the administration being thereafter discontinued. These animals exhibited an abbreviated rather than a prolonged duration of life.

Reverting to the experimental data presented in this paper it will be seen that while the long lived animals in any experimental group usually exhibited initial overgrowth in comparison with the short lived animals they displayed much less tendency to form late accretions of tissue. Bearing in mind the fact that the late growth of animals consists predominantly in connective tissue formation, these facts would appear to harmonize with the view that the potential longevity of any given individual is determined by the relative velocities of anabolism in the cellular and sclerous tissues. A high rate of cellular anabolism, while encouraging the growth of distinctively cellular tissues, defers the connective tissue accretion which is characteristic of senescence. A low rate of cellular anabolism renders available a higher proportion of nutrients for the distinctively structural tissues and necessitates premature senescence.

Since longevity is determined, not by the absolute mass of tissue, but by the relative proportion of parenchymatous to

[17] Chalatow, S. S., *Virchows Arch. path. Anat.*, 1912, ccvii, 452; *Beitr. path. Anat. u. Path.*, 1914, lvii, 85. Anitschkow, N., *Beitr. path. Anat. u. Path.*, 1913, lvi, 379; 1914, lvii, 201. Weltmann, O., and Biach, P., *Z. exp. Path. u. Therap.*, 1913, xiv, 367.

sclerous tissues, there is no single magnitude or contour of growth curve which is characteristic of long lived animals, and for the same reason the longevity of different species of animals bears no relation to their mass or bulk. On the other hand the longevity of different species has been shown to stand in definite relation to the proportionality between different types of tissue; *e.g.*, of brain weight to total body weight.[18]

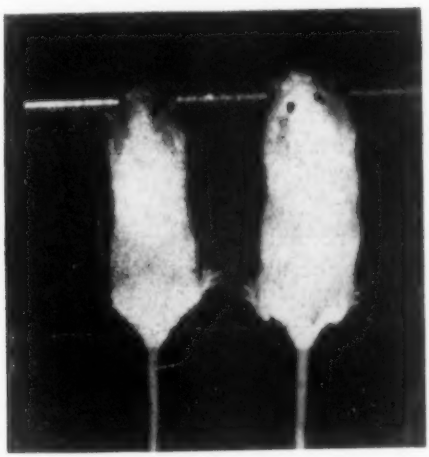

FIG. 3. Photograph of two female mice, each 500 days old. The smaller animal (25 gm.) is the average weight of a normal female of this age. The larger (31 gm.) has received a brief preadolescent administration of tethelin (8th to 12th weeks) which was thereafter discontinued. Its weight is the average weight of twenty-four animals similarly treated. The discontinuation of the tethelin before sexual maturity has permitted the actual supernormality of growth induced by this agent to express itself by the reestablishment of the normal proportion of sclerous to parenchymatous tissues which the continuous administration of tethelin prevents.

SUMMARY.

1. In any given group of animals those which live longest represent a relatively stable subgroup, highly resistant to external disturbing factors, displaying subnormal variability and a more or less marked, but not invariable, tendency to early overgrowth and relative paucity of tissue accretion in late life.

[18] Friedenthal, H., *Centr. Physiol.*, 1910 II, xxiv, 321.

2. The short lived animals, on the contrary, are relatively unstable, sensitive to external disturbing factors, supernormally variable and, as a rule but not invariably, display relatively deficient early growth and a tendency to rapid but unstable accretions of tissue in late life.

3. The hypothesis is presented that the potential longevity of any given individual is determined by the relative velocities of anabolism in the cellular and sclerous tissues respectively.

4. In terms of this hypothesis it is suggested that the prolongation of life which is brought about by continuous administration of tethelin to mice is attributable to an increased velocity of anabolism of distinctively cellular elements to the relative disadvantage, in the competition for nutrients, of the connective tissue elements.

5. If the administration of tethelin is discontinued prior to sexual maturity the normal proportion of cellular to connective tissues is rapidly restored and exceptionally large animals of normal or abbreviated duration of life are the result.

TABLE I.

Normal Males.

(Average duration of life 767 days.)

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
4	10.50	12.38		24.4
5	11.05	12.45	20.1	24.6
6	15.39	15.58	22.7	22.0
7	17.25	18.57	17.6	16.9
8	18.28	20.02	14.9	15.9
9	19.38	21.40	18.4	16.5
10	20.03	21.90	17.6	16.7
11	20.75	22.46	14.4	13.3
12	21.84	23.15	14.5	13.7
13	22.34	24.05	15.8	14.2
14	22.81	24.88	14.6	14.1
15	23.34	25.03	12.8	11.9
16	23.78	25.53	13.2	12.7
17	24.00	26.18	13.2	12.4
18	24.84	26.23	13.8	12.1
19	24.69	26.76	13.0	12.1
20	25.28	26.79	12.4	10.8
21	25.50	26.95	12.2	11.2
22	25.31	26.68	10.6	9.0
23	25.47	27.03	11.6	9.8
24	26.09	27.47	11.6	10.1
25	26.03	27.89	12.1	11.0
26	26.06	27.76	10.7	10.8
27	25.33	27.62	12.6	11.3
28	26.27	28.06	11.0	10.9
29	25.90	28.19	10.3	10.9
30	26.50	27.91	9.8	9.5
32	26.97	28.25	9.8	9.6
34	27.00	28.47	9.9	10.5
36	27.31	28.68	10.2	10.1
38	27.41	29.11	10.3	10.5
40	27.28	29.39	9.6	10.4
42	27.34	29.36	10.7	11.2
44	27.50	29.89	12.0	12.0
46	27.75	29.46	11.7	11.7
48	27.44	29.11	10.5	10.8
50	27.97	29.93	11.3	11.1
52	27.66	29.86	10.1	10.7
54	27.47	30.04	11.6	12.0

TABLE I—*Concluded.*

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
56	28.28	30.50	11.8	12.0
58	28.34	29.96	11.3	10.9
60	28.13	30.18	11.4	11.8
62	28.47	30.50	10.8	11.6
64	28.53	30.68	10.2	11.4
66	28.56	31.00	10.2	11.4
68	28.72	31.04	10.0	11.1
70	28.81	30.71	9.9	10.9
72	28.59	31.00	11.5	12.2
74	28.84	30.92	9.8	10.9
76	29.28	30.85	9.4	10.0
78	29.13	31.42	9.5	11.5
80	29.25	30.81	10.4	11.1
82	29.59	31.45	9.7	11.3
84	29.28	30.85	10.7	12.3
86	29.44	31.28	10.4	13.0
88	29.19	32.19	11.1	13.0
90	29.25	31.88	10.8	11.3
92	29.28	32.81	9.0	12.0
94	29.28	31.81	9.6	11.1
96	29.25	30.64	9.6	10.1
98	29.00	30.33	9.6	10.0
100	28.91	31.60	8.6	9.6
102	28.75	32.38	10.3	10.7
104	29.19	31.83	9.7	10.4

Summary of Table I.—The long lived animals form a group which are initially lighter than the short lived animals and do not grow more rapidly so that the relative subnormality is maintained throughout life. They are less variable than the short lived animals initially and after the 30th week, but between the 9th and 30th weeks they are more variable than the short lived group.

TABLE II.

Normal Females.

(Average duration of life 719 days.)

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
4	11.21	10.39	23.9	23.4
5	13.64	11.81	21.2	19.4
6	15.50	14.12	17.7	18.9
7	17.17	16.39	15.8	15.0
8	18.14	17.83	14.4	13.9
9	18.64	18.92	14.3	13.4
10	19.31	19.44	15.0	14.3
11	20.03	20.05	13.4	12.6
12	20.31	20.31	13.6	12.6
13	20.89	21.19	13.8	13.0
14	21.28	21.14	15.0	13.2
15	21.78	21.78	14.2	13.7
16	22.00	22.28	13.2	12.6
17	22.28	22.31	13.2	11.9
18	22.47	21.97	13.2	11.9
19	22.67	22.53	12.9	12.0
20	22.50	22.70	12.8	11.2
21	23.19	22.92	13.6	12.3
22	23.28	23.36	14.2	12.5
23	23.59	23.44	14.1	12.0
24	23.67	23.69	13.0	11.3
25	23.82	23.75	13.0	11.4
26	24.08	24.00	12.0	11.6
27	23.68	24.31	13.8	11.8
28	23.64	23.53	11.9	10.3
29	24.00	23.69	13.4	12.2
30	23.92	23.92	14.0	12.4
32	24.25	24.11	13.6	11.5
34	24.19	24.17	13.8	12.2
36	24.75	24.56	13.0	11.2
38	25.17	24.41	14.1	12.3
40	25.50	24.53	14.1	11.9
42	25.58	24.53	15.6	13.1
44	26.06	24.91	17.7	15.3
46	26.31	24.93	16.6	14.6
48	26.08	24.64	16.4	14.6
50	26.03	24.82	15.8	14.0
52	26.22	25.00	16.9	15.3
54	26.47	24.64	17.4	15.8

TABLE II—*Concluded.*

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
56	26.67	24.91	17.8	16.5
58	26.94	25.14	17.9	16.7
60	27.00	24.68	18.6	17.8
62	27.28	24.95	19.2	17.7
64	27.58	25.09	18.8	17.8
66	27.50	24.55	19.8	19.2
68	27.58	24.70	18.5	18.3
70	27.22	23.94	18.9	18.7
72	27.36	24.75	18.4	17.7
74	27.42	25.07	17.8	16.9
76	27.83	25.71	18.7	17.7
78	27.86	25.70	17.6	17.0
80	27.92	26.38	18.4	17.4
82	27.69	26.00	17.9	17.0
84	27.89	27.13	17.2	16.4
86	28.00	27.38	18.9	17.6
88	27.92	27.13	19.8	18.7
90	27.97	27.13	19.2	16.8
92	28.08	27.33	19.3	18.4
94	27.81	25.00	18.5	18.0
96	28.31	25.50	18.4	17.8
98	28.22	22.00	20.6	20.9
100	27.86	19.50	21.4	22.2

Summary of Table II.—The long lived animals are initially heavier than the short lived animals. From the 9th till the 36th weeks the weights of the two groups are approximately equal. Thereafter the long lived animals grow more rapidly than the short lived animals so that by the 70th week they exceed the short lived animals by over 3 gm. The variability of the long lived group slightly exceeds that of the short lived group until the 66th week, after which the variabilities are approximately equal. Death in the short lived group is preceded (70th week onward) by a sharp rise of weight.

TABLE III.

Pituitary-Fed Males.

(Average duration of life 792 days.)

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
4	12.13	13.21	13.7	22.7
5	12.72	13.45	19.0	24.1
6	15.59	16.08	18.8	22.1
7	17.28	17.45	15.8	17.7
8	18.72	18.50	17.4	17.3
9	19.22	19.80	15.4	14.8
10	20.03	20.03	15.2	15.4
11	20.66	19.53	15.3	14.9
12	21.84	21.75	9.4	11.4
13	22.06	22.60	8.4	10.7
14	22.91	23.08	8.0	10.0
15	22.88	23.20	7.4	9.8
16	23.66	23.68	7.6	9.3
17	24.10	24.12	8.2	9.9
18	24.25	24.10	8.1	9.7
19	24.44	24.68	9.0	10.2
20	24.44	24.78	8.5	9.8
21	25.06	24.93	8.4	9.6
22	25.47	25.25	9.8	11.5
23	25.91	25.80	7.7	8.7
24	25.97	26.38	7.7	10.5
25	25.81	26.00	9.1	9.8
26	25.72	26.43	7.6	9.2
27	26.00	26.20	7.9	8.8
28	26.17	26.39	7.3	8.7
29	26.06	26.37	8.1	8.8
30	26.21	26.68	9.2	8.4
32	26.38	26.93	8.3	9.0
34	26.81	27.83	8.7	9.7
36	26.78	27.88	7.6	9.3
38	27.00	28.72	8.4	9.3
40	27.34	28.72	7.7	9.2
42	27.47	28.67	8.6	10.6
44	27.53	29.17	8.1	10.0
46	27.53	29.00	8.3	10.1
48	27.13	29.11	8.7	11.1
50	27.53	29.54	8.8	11.2
52	27.66	29.64	8.1	11.2
54	28.09	29.86	9.6	11.4

TABLE III—*Concluded.*

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
56	28.06	29.86	8.4	11.1
58	28.50	30.32	8.7	11.0
60	28.44	30.54	8.4	11.0
62	28.19	30.18	7.8	10.6
64	28.19	30.57	7.8	11.3
66	28.75	31.00	7.6	11.4
68	28.88	30.50	8.0	11.4
70	28.41	30.46	8.0	11.9
72	28.28	30.96	8.3	11.0
74	28.72	30.88	9.0	11.5
76	28.25	30.50	8.0	11.2
78	28.63	30.55	9.4	10.2
80	28.22	30.18	7.7	9.8
82	28.22	30.23	7.6	10.1
84	28.78	30.14	8.0	10.2
86	28.00	29.15	7.5	8.2
88	28.50	29.39	8.1	9.0
90	28.81	29.06	8.3	8.6
92	28.03	28.06	7.3	6.5
94	28.44	28.44	7.8	7.9
96	28.38	28.13	8.0	7.8
98	28.06	28.43	8.4	8.4
100	27.94	28.93	7.5	8.1
102	28.38	28.10	7.5	9.2
104	27.78	28.30	7.5	7.5
106	27.97	28.10	6.6	8.1
108	27.97	27.75	6.8	6.7
110	28.00	26.50	7.1	7.6
112	28.04		8.2	6.3

Summary of Table III.—The long lived animals are initially lighter than the short lived animals, but rapidly gain on them, so that by the 10th week the two groups are equal in weight. The long lived never surpass the short lived animals, however, and by the 24th week begin to drop behind and by the 40th week and thereafter are markedly lighter than the short lived group. The long lived animals are consistently much less variable than the short lived animals.

TABLE IV.

Pituitary-Fed Females.

(Average duration of life 704 days.)

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
4	9.84	9.04	18.5	20.5
5	12.28	11.07	16.8	19.6
6	14.35	13.27	13.3	16.1
7	15.40	13.73	13.8	15.8
8	16.79	15.47	12.4	15.1
9	17.64	16.63	11.0	13.1
10	18.40	17.27	11.0	12.1
11	18.52	17.83	8.7	10.5
12	19.19	18.43	7.7	9.3
13	19.57	18.70	7.8	10.3
14	19.95	19.03	7.3	9.4
15	19.93	19.43	9.4	10.4
16	20.17	19.53	7.7	9.3
17	20.83	20.00	7.6	9.6
18	21.07	20.23	7.7	9.7
19	21.45	20.77	7.9	9.7
20	21.52	20.77	7.5	9.1
21	21.58	21.03	8.6	9.6
22	21.90	21.33	8.7	10.2
23	21.85	21.43	8.0	9.9
24	22.40	21.60	8.6	10.2
25	22.30	21.70	8.3	10.4
26	22.43	21.63	9.1	10.5
27	22.58	21.90	8.8	10.3
28	22.98	21.93	8.0	10.0
29	22.75	21.90	8.4	10.3
30	23.05	22.71	8.3	9.6
32	23.50	22.47	8.8	10.8
34	23.81	22.87	9.1	10.8
36	24.14	22.90	9.7	10.9
38	24.29	23.63	11.2	12.2
40	24.74	23.86	11.4	11.8
42	24.88	23.83	11.6	12.4
44	25.12	23.81	11.8	13.1
46	25.19	23.85	11.0	13.2
48	25.26	23.88	12.3	13.7
50	25.81	24.69	12.6	13.3
52	26.40	24.57	15.2	15.4
54	26.05	25.42	14.8	15.2

TABLE IV—*Concluded.*

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
56	26.60	25.13	14.9	15.0
58	26.57	25.71	16.1	15.9
60	26.90	26.10	13.2	14.5
62	27.12	25.80	15.1	16.0
64	26.81	25.75	14.8	16.3
66	27.19	26.61	16.1	16.8
68	27.33	27.19	16.4	16.9
70	27.24	26.01	16.2	17.0
72	27.02	26.75	15.6	16.6
74	27.26	26.56	16.6	16.8
76	27.17	25.83	16.1	15.7
78	26.93	26.00	13.9	13.9
80	27.21	25.58	16.2	16.2
82	27.48	26.00	18.0	17.6
84	27.43	25.60	17.9	17.6
86	27.33	27.38	18.8	17.6
88	27.26	27.50	15.2	14.4
90	27.07	25.50	16.0	15.6
92	26.48	27.25	12.0	11.6
94	26.53	26.50	12.2	11.8
96	26.53	31.50	14.2	13.2

Summary of Table IV.—The long lived animals form a relatively invariable group, in which preadolescent growth is unusually rapid. The lead thus gained is maintained throughout life. The death of the short lived animals is preceded by a marked gain in weight accompanied by an increase in variability. The same increase in variability is noted at the same time in the long lived group, but the increase in weight is relatively less, and less subject to fluctuation than in the short lived group.

TABLE V

Tethelin-Fed Males; Continuous Administration.
(Average duration of life 866 days.)

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
4	13.36	12.80		
5	13.14	12.94	19.9	21.8
6	14.46	14.95	19.2	18.2
7	16.69	16.36	14.1	14.6
8	18.62	17.73	9.0	12.0
9	19.23	18.68	6.8	10.2
10	19.92	19.00	6.8	10.8
11	20.27	19.50	6.7	10.9
12	21.08	19.95	7.6	10.6
13	21.50	20.14	5.8	10.5
14	21.62	20.00	7.5	11.2
15	21.19	21.23	8.9	9.1
16	22.19	21.23	7.3	8.4
17	22.50	22.82	8.0	8.0
18	23.12	23.32	6.9	7.3
19	23.92	23.64	7.0	7.2
20	24.65	24.00	5.4	6.3
21	24.65	24.36	5.0	6.2
22	25.00	24.70	6.4	7.6
23	24.77	24.40	5.4	6.4
24	25.27	24.45	7.2	7.7
25	25.73	24.80	6.2	6.7
26	25.69	24.80	6.8	8.1
27	26.08	24.95	7.0	8.0
28	25.85	24.95	6.7	7.4
29	26.15	24.95	6.4	7.9
30	26.00	25.28	6.8	7.2
32	26.46	25.40	5.7	7.1
34	26.69	25.60	6.9	8.8
36	26.31	25.65	6.2	6.9
38	26.27	26.20	6.7	7.4
40	26.54	26.30	5.1	5.4
42	26.65	26.40	5.8	5.8
44	26.81	26.10	6.3	6.9
46	27.04	26.35	7.1	7.0
48	26.88	26.65	6.4	6.9
50	26.85	26.10	7.7	7.8
52	27.35	26.50	7.5	7.2
54	27.81	27.40	6.3	7.0

TABLE V—*Concluded.*

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
56	28.04	27.30	7.8	7.7
58	27.77	26.75	6.4	6.7
60	27.23	26.55	7.4	7.3
62	27.81	27.00	6.3	7.0
64	27.73	27.45	7.0	7.2
66	27.62	26.70	6.9	7.9
68	28.31	27.75	6.5	7.9
70	28.42	27.70	6.9	7.8
72	28.42	27.60	7.3	7.8
74	28.46	27.30	7.6	8.4
76	28.88	28.05	6.4	7.7
78	28.54	27.70	7.2	8.2
80	28.96	28.11	7.3	9.2
82	28.81	28.00	5.3	7.7
84	28.19	28.00	6.4	7.9
86	28.62	28.00	7.2	8.8
88	28.62	27.56	6.7	8.4
90	29.04	29.92	6.8	7.9
92	28.81	28.84	7.6	8.4
94	28.58	29.25	6.9	8.2
96	28.58	29.60	5.9	8.7
98	28.62	29.60	9.3	10.1
100	28.65	29.10	6.6	8.2
102	28.50	28.80	6.1	8.2
104	28.85	29.40	6.0	8.5
106	28.69	29.00	7.9	9.2
108	28.81	29.00	6.7	7.9
110	28.58	29.20	6.8	7.9
112	28.77	28.70	6.8	7.8
114	28.85	28.40	7.0	7.3
116	28.31	26.50	7.0	6.7

Summary of Table V.—The long lived animals form a relatively invariable group in which preadolescent growth is unusually rapid. The lead thus established is maintained in diminishing degree throughout life. Death of the short lived group is preceded by a marked gain in weight, both absolutely and relatively to the long lived group.

TABLE VI.

Tethelin-Fed Females; Discontinuous Administration 4th to 8th, 21st to 25th, and 42nd to 46th Weeks.

(Average duration of life 800 days.)

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
4	10.50	11.60	28.8	27.6
5	13.13	13.75	22.6	19.6
6	14.56	15.31	22.0	19.4
7	15.88	15.69	17.9	16.1
8	16.06	16.31	15.8	12.6
9	16.56	16.69	15.2	12.4
10	17.00	17.06	15.0	13.2
11	17.56	17.50	18.1	15.7
12	17.88	17.44	19.2	16.6
13	18.94	19.00	16.3	14.1
14	19.75	19.69	13.0	11.9
15	20.56	20.50	13.4	11.4
16	20.31	20.06	12.8	11.2
17	21.00	20.19	12.4	11.5
18	20.31	20.31	11.6	10.7
19	20.63	20.56	14.1	11.9
20	20.88	20.31	14.0	12.1
21	21.31	20.88	11.9	11.2
22	21.50	20.75	12.0	11.1
23	21.88	21.06	13.6	12.2
24	22.13	21.44	14.7	12.7
25	22.38	21.13	13.6	12.8
26	22.56	21.44	14.8	13.5
27	22.50	21.19	14.4	13.6
28	22.81	21.63	15.8	14.2
29	22.57	22.14	16.0	14.1
30	23.25	21.50	17.4	15.4
32	23.63	21.56	16.9	15.4
34	23.94	22.19	17.6	15.7
36	24.06	21.69	16.4	15.7
38	24.25	22.63	16.9	15.6
40	24.19	22.88	18.8	16.9
42	24.44	22.63	19.0	17.9
44	25.19	22.69	19.2	18.8
46	25.19	22.00	17.6	17.8
48	24.56	22.44	16.4	17.1
50	25.31	22.88	16.5	17.6
52	25.56	23.25	16.5	16.6

TABLE VI—*Concluded.*

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
54	25.38	23.44	18.5	17.2
56	25.38	23.75	16.6	17.1
58	25.50	23.25	16.7	16.7
60	25.56	23.06	16.7	16.8
62	25.38	23.19	15.6	16.8
64	25.50	23.19	15.2	16.7
66	25.13	23.19	14.5	16.1
68	25.19	22.94	13.8	14.7
70	25.00	23.13	14.0	15.4
72	25.88	24.14	15.4	16.3
74	25.31	24.00	14.3	15.5
76	25.63	24.07	14.5	15.7
78	25.25	24.14	14.1	15.5
80	25.38	24.07	15.3	16.4
82	24.50	23.29	14.4	16.0
84	25.13	23.00	14.4	16.6
86	25.94	22.80	13.9	16.1
88	25.63	23.60	15.5	17.9
90	25.25	23.40	12.6	16.8
92	24.25	26.67	14.4	15.1
94	24.69	27.33	12.0	12.5
96	24.50	26.25	13.6	12.6

Summary of Table VI.—The long lived animals are at first lighter and more variable than the short lived animals. From 23 weeks onwards, however, the long lived group not only catch up to but notably surpass the short lived group. At this stage they therefore grow more rapidly than the short lived animals. They remain more variable until the 44th week. Death, in the short lived group, is preceded by a small increase of weight at 72 weeks and at 92 weeks by a large and sudden increase which is in part but not wholly due to the death of the lighter individuals.

TABLE VII.

Tethelin-Fed Females; Brief Preadolescent Administration 4th to 12th Weeks Only.

(Average duration of life 695 days.)

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
4	10.90	10.31	7.9	9.0
5	13.35	11.77	18.8	20.0
6	14.27	13.50	14.8	16.2
7	16.12	14.86	13.8	15.9
8	17.00	16.46	13.0	15.0
9	17.81	16.55	13.6	14.8
10	18.27	16.77	12.5	14.2
11	19.08	17.50	11.7	13.4
12	20.00	18.75	13.0	13.6
13	20.62	19.00	10.5	11.8
14	21.15	19.50	13.2	13.3
15	21.15	19.95	12.6	12.6
16	21.77	20.10	11.8	11.7
17	21.92	20.85	11.4	11.4
18	22.65	21.10	11.4	12.0
19	23.00	21.80	11.4	12.1
20	22.77	21.45	10.8	11.9
21	23.23	22.00	12.2	12.7
22	23.35	21.80	11.4	12.4
23	23.38	22.05	10.9	12.1
24	23.65	22.65	12.0	12.3
25	23.88	22.65	12.9	13.0
26	24.35	22.75	11.8	12.6
27	24.12	22.75	14.0	14.3
28	24.35	22.60	13.6	15.1
29	24.04	22.85	12.4	13.3
30	24.88	22.85	13.4	14.7
No measurements were made between the 30th and 70th weeks.				
70	31.81	27.00	19.4	23.2
72	32.23	26.33	19.1	23.5
74	32.46	27.00	18.9	19.5
76	32.54	24.50	18.9	20.2
78	31.73	24.75	15.6	18.2
80	30.54	24.00	17.8	18.2
82	30.62	23.17	17.4	19.2
84	30.08	22.67	17.4	19.6

TABLE VII—*Concluded.*

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
86	29.85	21.50	17.1	20.7
88	29.38	25.00	16.8	16.9
90	29.38	25.25	17.4	17.4
92	28.92	26.50	17.8	17.4
94	28.81	25.50	17.8	17.6
96	28.15	25.00	16.4	15.8

Summary of Table VII.—The long lived animals form a group in which preadolescent growth is comparatively rapid and variability low. This superiority of growth is maintained and markedly enhanced in later ages, and the long lived animals remain the less variable until the average duration of life of the short lived group. The death of the short lived group may be preceded by a relatively sudden increase of weight (see the 70th to 74th weeks), but the lack of weighings between the 30th and 70th weeks renders it difficult to be sure of this. A sharp decrease of weight follows and slightly, but only slightly, precedes a similar loss of weight in the long lived group.

TABLE VIII.

Cholesterol-Fed Males.

(Average duration of life 764 days.)

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
5	12.83	12.85	19.2	18.1
6	13.98	14.19	17.0	19.3
7	15.75	16.00	14.2	16.2
8	16.80	17.00	13.7	15.5
9	16.85	16.71	16.4	17.9
10	17.38	17.46	17.6	18.4
11	17.88	17.82	16.9	18.2
12	19.23	18.57	14.2	16.1
13	20.70	20.11	11.5	13.5
14	21.88	20.64	10.4	12.4
15	22.33	21.25	11.7	13.3
16	22.33	21.61	10.3	12.9
17	22.34	22.00	9.4	11.0
18	23.25	22.89	10.5	11.5
19	23.05	21.81	7.6	9.9
20	23.90	22.61	8.2	11.0
21	23.67	23.17	7.0	9.1
22	24.58	23.64	9.6	10.5
23	24.03	24.00	7.3	9.4
24	24.56	24.23	9.7	10.3
25	24.24	24.25	6.9	8.9
26	24.64	24.23	9.2	10.5
27	24.12	24.21	6.8	9.2
28	25.22	24.64	9.2	10.4
29	24.35	24.92	7.2	9.1
30	24.82	24.67	10.5	11.2
32	25.38	25.29	10.1	11.1
34	25.53	25.75	10.5	11.3
36	25.88	25.36	11.0	11.8
38	26.08	26.35	9.8	9.9
40	26.07	26.04	12.7	11.8
42	26.38	25.88	11.0	11.5
44	26.03	26.67	10.1	10.4
46	27.25	26.58	9.8	11.7
48	26.90	26.73	8.6	9.4
50	27.05	26.46	8.5	9.3
52	26.85	26.67	9.4	10.5
54	27.63	27.29	8.6	9.9
56	26.93	27.08	9.1	9.8

TABLE VIII—*Concluded.*

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
58	26.75	26.83	9.8	11.3
60	26.93	26.79	9.5	10.8
62	27.05	27.08	9.2	10.1
64	27.08	26.83	8.5	10.2
66	27.03	26.83	9.5	10.8
68	26.93	27.29	8.6	10.9
70	27.23	27.23	8.3	10.2
72	27.15	27.36	9.5	10.9
74	27.33	27.27	8.6	11.1
76	27.33	28.17	9.5	10.8
78	27.03	26.72	10.2	11.1
80	27.25	27.78	9.2	10.3
82	27.35	28.63	9.6	10.9
84	26.83	28.00	10.5	11.8
86	26.98	28.14	10.8	12.4
88	27.43	28.36	10.4	11.3
90	27.15	27.79	11.0	12.3
92	26.83	27.50	10.2	12.1
94	26.60	27.42	12.0	13.7
96	26.53	27.75	13.2	13.0
98	26.43	26.60	12.0	13.4
100	26.28	25.83	13.4	14.4
102	26.53	25.33	12.2	13.9
104	26.25	28.25	12.4	13.6
106	26.65	22.00	12.0	12.4

Summary of Table VIII.—The long lived animals are initially smaller but soon thereafter grow more rapidly than the short lived animals and are less variable. The superiority of the long lived group is transient and disappears at about 25 weeks. Death of the short lived group is preceded by a relatively sudden increase in weight (80th week), the long lived animals displaying a simultaneous increase of variability but no increase in weight.

TABLE IX.

Cholesterol-Fed Females.

(Average duration of life 658 days.)

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
5	14.08	12.44	18.6	21.5
6	13.22	13.00	14.4	15.6
7	14.13	13.71	15.6	16.5
8	15.09	14.74	13.9	15.4
9	15.25	14.97	17.4	17.8
10	15.75	15.37	13.8	15.2
11	16.56	15.63	15.6	18.1
12	17.53	16.74	13.6	16.1
13	18.22	17.32	14.9	16.9
14	18.94	18.05	14.1	14.7
15	18.94	18.63	13.0	13.7
16	19.59	18.89	12.2	13.3
17	19.88	18.76	12.5	12.8
18	20.19	19.19	12.4	12.8
19	19.94	19.35	11.8	12.3
20	20.44	19.83	12.4	12.1
21	20.84	19.68	12.8	12.9
22	20.59	20.14	12.2	12.4
23	20.88	20.12	12.4	12.4
24	21.00	20.64	15.2	14.3
25	21.13	20.76	14.6	14.5
26	21.33	21.17	14.4	14.7
27	21.53	21.12	13.3	14.8
28	21.60	21.53	14.9	15.7
29	21.69	21.56	12.0	15.2
30	22.00	22.13	15.6	16.6
32	21.94	22.21	13.6	17.3
34	22.16	22.41	13.5	18.4
36	22.22	22.59	13.6	19.9
38	22.75	23.03	13.8	20.4
40	22.69	23.06	13.6	21.0
42	22.66	23.17	14.3	22.8
44	22.81	23.86	13.4	23.1
46	23.75	24.61	13.6	23.0
48	23.53	24.27	13.3	23.4
50	23.63	24.17	12.2	23.0
52	23.44	24.95	12.9	22.4
54	23.97	25.54	13.0	21.9
56	23.69	25.18	13.9	23.1

TABLE IX—*Concluded.*

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
58	23.38	25.35	13.7	23.0
60	23.84	25.15	14.0	21.1
62	24.16	25.56	12.6	22.3
64	23.94	25.17	13.4	21.7
66	23.97	24.83	14.1	21.6
68	24.25	23.14	14.8	15.5
70	24.34	22.79	15.0	15.5
72	24.66	23.29	16.3	16.9
74	24.38	23.29	14.3	15.8
76	24.19	23.29	16.1	16.8
78	24.13	23.57	14.7	16.0
80	24.50	22.83	16.9	17.9
82	24.75	21.75	16.4	18.5
84	24.69	21.33	16.8	19.1
86	24.44	21.00	16.4	19.3

Summary of Table IX.—Prior to the 30th week the long lived animals grow more rapidly than the short lived animals and they are less variable. The unusually early death of the short lived animals in this group is preceded by a remarkable gain of weight which enables them at a comparatively early stage to catch up to and surpass the long lived animals. This increase in weight is accompanied by an extraordinary concurrent increase of variability which is not perceived in the long lived group until they have attained over double the age (60 weeks). This confirms the view expressed in previous publications that the high variability of cholesterol-fed animals is due to deleterious effects of the cholesterol, since those animals which suffered from these effects most markedly also displayed the enhanced variability at an early age.

TABLE X.

Lecithin-Fed Males.

(Average duration of life 731 days.)

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
4	10.36	11.18	9.6	23.1
5	10.22	12.58	20.2	23.2
6	11.53	12.63	19.1	21.7
7	13.72	16.15	18.8	19.4
8	14.69	18.05	18.9	18.5
9	16.16	18.93	16.6	15.8
10	17.56	20.38	13.8	13.7
11	18.53	21.28	9.1	11.4
12	19.38	21.40	7.6	10.2
13	19.91	22.05	8.2	9.8
14	20.38	22.38	6.6	9.4
15	20.84	23.23	7.2	9.9
16	21.13	23.10	7.0	8.9
17	21.47	23.50	7.2	8.5
18	22.22	24.08	6.6	8.7
19	22.81	24.45	5.9	7.5
20	23.34	24.60	6.5	8.0
21	23.34	24.83	6.3	8.2
22	23.75	25.75	5.6	7.6
23	23.50	25.43	5.5	7.9
24	23.44	25.23	6.4	8.7
25	23.84	25.08	6.6	8.6
26	23.94	26.02	5.1	8.4
27	24.09	25.75	5.1	7.4
28	24.19	26.16	5.9	8.8
29	24.37	26.03	5.3	8.8
30	24.50	26.71	5.9	8.8
32	24.94	26.93	6.5	8.8
34	25.19	27.23	5.5	8.5
36	25.41	28.08	5.0	8.8
38	25.47	28.00	5.2	8.8
40	26.00	27.97	5.3	8.3
42	26.00	28.38	5.4	9.5
44	25.72	28.50	5.7	10.0
46	25.88	28.38	5.4	10.1
48	25.88	29.06	5.1	9.1
50	25.81	29.17	5.1	9.7
52	26.47	29.13	6.2	9.3
54	26.91	29.39	5.2	8.7

TABLE X—*Concluded.*

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
56	26.59	29.21	5.8	9.0
58	26.97	29.04	5.4	7.8
60	26.84	28.96	6.2	8.1
62	26.50	28.83	4.9	7.5
64	26.94	29.25	4.9	7.6
66	26.81	29.25	5.7	7.4
68	26.53	28.77	5.3	7.3
70	27.16	28.85	6.4	7.8
72	27.16	28.56	5.2	6.4
74	26.78	29.06	6.1	8.1
76	26.94	28.63	5.7	7.6
78	27.00	27.75	4.1	5.1
80	27.22	28.50	5.5	6.5
82	26.75	28.25	6.9	7.5
84	26.66	27.83	5.0	6.0
86	26.47	29.13	6.0	6.7
88	26.91	28.83	5.8	6.0
90	27.22	28.75	6.4	6.4
92	27.25	28.25	5.4	5.2
94	26.84	28.00	6.7	6.6
96	26.63	26.63	6.8	6.8

Summary of Table X.—The long lived animals are initially lighter than the short lived group. This relative subnormality is enhanced with time and amounts by the 40th week to 2.5 gm. They are consistently and very decidedly less variable than the short lived group. The short lived animals display a marked increase of weight between the 30th and 50th weeks, death being preceded by a gradual slight loss of weight.

TABLE XI.

Lecithin-Fed Females.

(Average duration of life 677 days.)

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
4	9.44	8.00	14.7	16.3
5	11.89	10.85	16.3	15.8
6	13.58	13.09	14.3	12.7
7	14.97	15.12	12.3	9.7
8	16.34	16.62	9.5	8.8
9	16.79	17.24	9.4	8.6
10	17.24	17.41	10.0	9.6
11	18.00	18.48	9.0	8.2
12	18.37	19.00	8.6	8.6
13	18.42	19.06	9.0	8.2
14	18.89	19.69	7.9	7.6
15	19.50	19.97	9.7	8.9
16	19.87	20.22	8.3	8.4
17	20.03	20.53	8.7	8.5
18	20.13	20.94	9.0	9.0
19	20.45	21.16	8.7	8.6
20	20.32	21.03	8.5	8.5
21	20.71	21.41	9.7	8.8
22	20.76	21.56	9.9	9.1
23	21.03	21.66	9.3	9.0
24	20.97	21.63	9.5	9.4
25	21.03	21.72	10.4	9.4
26	21.47	21.88	9.8	8.9
27	21.29	22.00	10.2	9.8
28	21.76	22.09	10.7	9.8
29	21.79	22.38	10.4	10.2
30	22.18	22.50	10.2	10.6
32	22.16	22.47	11.6	11.2
34	22.32	22.89	10.7	10.9
36	22.61	23.25	10.4	10.8
38	22.47	23.29	11.7	11.4
40	22.74	23.29	11.3	11.2
42	22.82	23.25	13.2	12.1
44	22.97	23.71	12.8	12.9
46	22.74	23.88	14.6	13.6
48	23.39	24.92	13.4	14.2
50	23.34	24.08	14.7	14.4
52	24.13	24.60	13.8	14.0
54	23.84	25.90	14.0	14.7

TABLE XI—*Concluded.*

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
56	23.63	25.50	14.2	14.1
58	24.00	26.00	13.9	13.5
60	23.87	24.56	14.3	13.1
62	23.39	24.44	13.4	12.4
64	24.26	24.50	14.6	12.9
66	24.84	24.78	14.0	13.1
68	23.74	24.11	15.4	14.4
70	24.53	24.81	14.8	14.4
72	24.13	20.50	14.3	15.0
74	24.29	25.17	15.0	14.2
76	24.66	25.50	15.3	14.5
78	24.71	26.33	15.2	14.3
80	24.45	26.50	15.4	14.4
82	24.39	26.33	15.3	14.4
84	23.97	26.25	15.0	14.5
86	24.08	26.50	16.6	15.8
88	24.24	27.25	16.3	15.7
90	24.47	26.00	18.0	17.6

Summary of Table XI.—The long lived animals are initially heavier and less variable than the short lived animals, but from 8 weeks onward the short lived animals are slightly heavier than the long lived animals, the variability of the two groups being the same.

TABLE XII.

Experimental group.	Duration of life.				Difference as per cent of average.
	Average.	Long lived.	Short lived.	Difference.	
	days	*days*	*days*	*days*	*per cent*
Males:					
Tethelin	866	979	718	261	30
Pituitary	792	926	649	277	35
Normal	767	928	606	322	42
Cholesterol	764	889	592	297	39
Lecithin	731	943	519	424	58
Females:					
Tethelin	800	970	630	340	42
Normal	719	863	533	330	46
Pituitary	704	831	514	317	45
Tethelin (brief administration)	695	867	472	395	57
Lecithin	677	829	484	345	51
Cholesterol	658	830	486	344	52

SOME OBSERVATIONS ON COLORIMETRIC ESTIMATIONS WITH SOLUTIONS CONTAINING TWO COLORED SUBSTANCES.

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1. INTRODUCTION.

Colorimetric methods for the estimation of small quantities of various substances have been developed to a considerable extent in recent years. At present, such methods appear to offer the most promising future for the quantitative determination of certain constituents present in small amounts in blood. The advances in these methods have been remarkable, although at times their accuracy and reliability have been somewhat overestimated. The general principle of such methods involves the reaction of the constituent to be determined with an added substance to produce a colored substance in a definite chemical ratio, and the estimation of the amount of colored substance formed by colorimetric comparison with a known amount of standard substance. In such methods, a colored substance may be produced from a (to the eye) colorless substance, as in the uric acid method of Folin and Denis[1], or one colored substance may be converted into a differently colored substance. The latter method evidently suggests possibilities of confusing complexity. The glucose method of Lewis and Benedict[2] applied to blood and of Dehn and Hartmann[3] depends upon the production of a red to brown reduction product, probably picramate, from a yellow picrate

[1] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912–13 xiii, 469.

[2] Lewis, R. C., and Benedict, S. R., *Proc. Soc. Exp. Biol. and Med.*, 1913–14, xi, 57; *J. Biol. Chem.*, 1915, xx, 61.

[3] Dehn, W. M., and Hartmann, F. A., *J. Am. Chem. Soc.*, 1914, xxxvi, 403.

under definite conditions. This method was developed further by Benedict and Osterberg[4] so as to make possible the determination of the glucose (or reducing substances) present in ordinary "normal" urines. Because of some apparently contradictory results obtained by different workers with this method, a careful study was made of simple conditions involved in this reaction. Certain phases of the results which are presented here are given in some detail because of their general interest and applicability to all colorimetric measurements.

2. *Significance of Colorimetric Comparisons.*

Some space may be devoted to the physical significance of the readings obtained with colorimeters in general use. When two solutions are matched in these, they have the same color. They need not be identical in composition or content. A color, as determined by the eye, does not fix the wave-lengths in the spectrum given by the substance. It is frequently stated that the wave-lengths of the spectrum of a substance determine its color. This statement requires a significant reservation. If two different wave-lengths are shown in the spectrum of a substance, then the color of the substance, for example in solution by transmitted light, will have a certain value to the eye. By color value is meant the physiological sensation which is considered to be produced by color and intensity of illumination. It is difficult to form a mental picture of these two properties separately, but it can readily be understood that both are involved in color sensation. For want of a better term, color value is used here for this phenomenon. If the concentration of the substance in the solution is halved, then doubling the length of the column of solution would give the same color value to the eye, since the light passes through the same amount of colored substance. The assumed two wave-lengths of the spectrum possess certain intensities which take part in determining the color value observed. A second substance will now be taken which at a certain concentration in solution is matched in a colorimeter against the first. The spectrum of the second substance is assumed to give two wave-lengths differing from those of the first substance, but the colors of the two substances as seen by the eye are the same at certain

[4] Benedict, S. R., and Osterberg, E., *J. Biol. Chem.*, 1918, xxxiv, 195.

concentrations. Varying the concentration of the second substance in solution will give comparable results when matched against itself, as does the first, but colorimetric comparisons of different concentrations of the two substances may give irregular results because the intensities of the different sets of wave-lengths may be different and show different variations on dilution. The color values are determined by both the wave-lengths and their intensities, so that variations in the latter may result in unexpected color values as determined by the eye for solutions which might be expected to be similar.

The hypothetical case just outlined is made actual by the colorimetric comparisons of bichromate and picramate solutions, the results of which are given in Section 5 *d*. It would appear that this relation applies and must be taken into account whenever two solutions containing differently colored substances are compared colorimetrically.

Identity of color as determined visually is the basis of the use of colorimeters such as the Duboscq. Such a color may be made up of two or more colored substances or of a pure substance, and still show visual identity with a different substance. This leads to another conclusion. Very few eyes are exactly similar in matching color, so that with mixed colored substances differences may be observed by different individuals. Such differences are less likely to occur with a substance of a definite color matched against itself in different concentrations. The colorimeter does not compare differences in color. When a match is obtained, the colors are assumed to be identical. The intensity of illumination of the two fields in a colorimeter gives a measure of the relative concentrations of the two solutions being compared. Very small differences in intensity of illumination, or amount of light per unit of surface, or brightness, can be observed. In practical work, the lengths of the columns of the liquids are varied until the fields are identical in intensity. The heights of the columns through which the light passes give then the relative concentrations of the dissolved substance in the two solutions. The light in the two cases is assumed to pass through the same absolute amounts of colored substance. This assumes Beer's law, that the amount of absorption is proportional to the concentration of dissolved substance. Comparing different concentrations of a

solution of one colored substance, this law holds in all probability for the ordinary uses to which a colorimeter is put. It is obvious, however, that complications may result as a consequence of assuming the validity of this law when comparing solutions of the same visual color at various concentrations, or when the colors are due to different substances or to the (visual) combination of two colored substances in varying proportions in the different solutions which are compared. Also the nature of the source of light may influence colorimetric readings, so that the exact illumination used should be stated in every colorimetric study.

3. Experimental Conditions in the Benedict Method.

After removal of interfering substances from the urine, or similar solution, Benedict and Osterberg determine the glucose, or reducing substance, by the partial reduction by heat in a carbonate solution of a yellow picrate to a red product, using as a standard for color comparison a glucose solution treated similarly or a picrate-picramate solution, or if picramate is not available a bichromate solution. The variable factors in the unknown solutions compared colorimetrically are the concentrations of picrate, of alkali, and of picramate. The lengths of time of heating to develop the colors may also be varied. In the final unknown solutions, the content of picrate (calculated as picric acid) is 5.8 mg. or 11.5 mg. per cc., the latter with the more dilute glucose solutions, the former with the more concentrated. These figures are not corrected for the amounts of picrate reduced, but these amounts are negligible. The amount of sodium carbonate added to bring about the reaction to produce the color is the same in every case, but before colorimetric comparison the solutions are diluted with water so that finally the concentrations of the sodium carbonate added may be in the ratio 2 to 1 in different solutions. The concentration of picramate or reduced substance will obviously vary with the concentration of glucose or reducing substances. As for the time of heating, it was originally stated that 10 minutes were satisfactory. It was found later that this was insufficient, but the most satisfactory length of time was not fixed. Greatly as a result of the color comparisons, Benedict considered the production of picramate to be the reaction taking place.

4. Experimental Method.

A Duboscq colorimeter with cups 52 mm. deep and 20 mm. inside diameter, and plungers 113 mm. in length was used in this work. The colorimeter was enclosed in a box the inside walls of which were painted with white paint. A National Mazda (Western Electric Sunbeam) 75 watt, 120 volt lamp, the direct rays of which were screened from the colorimeter by a partition, was used. Indirect constant illumination was obtained in this way. Heating was avoided by having a slit in the rear of the box for ventilation and by cutting off illumination when readings were not being made. All the precautions ordinarily observed in the use of a colorimeter were taken. Every result given was the average of from four to eight readings.

The picric acid used was purified and tested by the method of Folin and Doisy.[5] With a saturated solution of picric acid set at 20 mm. in the colorimeter, an average reading of 13.3 mm. was given by 20 cc. of this solution to which 1 cc. of 10 per cent sodium hydroxide solution had been added and allowed to stand for 15 minutes. After 24 hours, no change could be detected.

Picramic acid was prepared from picric acid and ammonium sulfide, and crystallized from acetic acid and then from water. It melted sharply at 168° (corrected).

The glucose used was the purest commercially obtainable and was dried before being used. Made up to a 4 per cent solution, it showed a rotation of 4.14° in a 200 mm. tube at 25° with sodium light (calculated 4.20°).

5. Experimental Results.

In order to obtain as complete a picture as possible of the conditions involved in the picrate-sugar method, the following series of experiments were carried out:

(*a*) Picrate against picrate (varying concentrations and alkalinities).

(*b*) Bichromate against bichromate (varying concentrations).

(*c*) Picramate against picramate (varying concentrations and alkalinities).

[5] Folin, O., and Doisy, E. A., *J. Biol. Chem.*, 1916-17, xxviii, 352.

(*d*) Picramate against bichromate.

(*e*) Picramate plus picrate against picramate plus picrate (varying concentrations).

(*f*) Glucose-picrate product against picramate plus picrate (varying concentrations).

(*a*) *Picrate against Picrate (Varying Concentrations and Alkalinities).*—The colors of picric acid solutions, even when saturated, were too faint for colorimetric study. The color of a picrate solution deepens with increase in concentration of sodium hydroxide as pointed out by Folin.[6]

With a solution to which had been added equivalent amounts of picric acid and sodium hydroxide matched against a similar solution as standard, addition of sodium hydroxide deepened the color, but addition of sodium carbonate did not until after a certain quantity had been added. For example, in a solution which contained per cc. (calculated in terms of the constituents added)[7] 14.4 mg. of picric acid and 2.5 mg. of NaOH, practically no change in color was observed by the addition of sodium carbonate until more than 9.6 mg. per cc. (or almost three equivalents per equivalent of picric acid) had been added.

The concentrations in the solutions used by Benedict are well within these limits. The picrate solution used in the development of the color contains approximately 36 mg. of picric acid per cc.; including the alkali, picric acid, and sodium hydroxide in the ratio of equivalents of nearly 1.25 to 1. The sodium carbonate added makes the final solutions, omitting the very small amount of picrate reduced in the reaction, have the following content per cc. when compared in the colorimeter:

When made up to a final volume of 25 cc.:
5.8 mg. of picric acid; 0.8 mg. of NaOH; 8.0 mg. of Na_2CO_3.
When made up to a final volume of 12.5 cc.:
11.5 mg. of picric acid; 1.6 mg. of NaOH; 16.0 mg. of Na_2CO_3.

(*b*) *Bichromate against Bichromate (Varying Concentrations).*—Accurate estimations of the content of potassium bichromate in

[6] Folin, O., *J. Biol. Chem.*, 1914, xvii, 475. Hunter A., and Campbell, W. R., *J. Biol. Chem.*, 1916–17, xxviii, 336.

[7] This method of stating the contents of a solution will be followed throughout this paper.

solution may be made by colorimetric comparisons. For example, halving the concentration was found to give twice the colorimeter reading at different settings.

(*c*) *Picramate against Picramate (Varying Concentrations and Alkalinities).*—Some experiments were performed with solutions of picramic acid, in which different concentrations were compared colorimetrically. The color values so determined were not proportional to the concentration of picramic acid. Also, exposure to light deepened the color of a picramic acid solution. Since picramic acid as such is not used in the method under investigation, these observations were not continued.

A number of solutions of picramate with different quantities of excess alkali were compared. Each solution was made up by

TABLE I.

Content.		Found.
Na_2CO_3		Picramic acid per cc.
per cent	*milli-equivalents per cc.*	*mg.*
0.8	150	0.098 (0.092–0.100)
1.6	300	0.098 (0.092–0.101)
3.2	600	0.098 (0.093–0.100)
6.4	1,200	0.100 (0.093–0.104)
NaOH		
2.5	625	0.104 (0.100–0.108)
5.0	1,250	0.104 (0.100–0.109)

dissolving 0.050 gm. of picramic acid in 500 cc. of the appropriate solution. Table I shows the results obtained when each was compared with all the others successively. 1 cc. of every solution contained 0.1 mg. or 0.0005 milli-equivalents of picramic acid.

The results in parentheses indicate the extremes of the averages of the comparison of the separate series with increasing alkalinity. With the carbonate solutions, the concentration of carbonate had no influence on the colorimetric comparisons. With sodium hydroxide solutions, there was apparently a slight increase in color, but not enough to introduce appreciable error. These alkaline solutions, exposed to the electric light for 24 hours, showed no difference in color, in contradistinction to the picramic acid solutions.

A strict proportionality was also found to exist between the colorimetric readings and concentration of alkaline picramate solutions with the sodium carbonate content recommended by Benedict and Osterberg.

(*d*) *Picramate against Bichromate.*—It was shown in Sections *b* and *c* that the color values, as determined colorimetrically, of bichromate solutions were proportional to the concentrations over wide ranges, and, similarly, that the color values of picramate solutions were proportional to the concentrations over wide ranges.

A number of bichromate and picramate solutions were compared colorimetrically. Table II shows some of the results of such a series.

TABLE II.

Bichromate.				Picramate Solution B.	
Solution A.		Solution B.			
Concentration per cc.	Colorimeter readings.	Concentration per cc.	Colorimeter readings.	Concentration as acid per cc.	Colorimeter readings.
mg.	*mm.*	*mg.*	*mm.*	*mg.*	*mm.*
15.0	15.0	15.0	15.0	0.042	18.5 (1)
15.0	15.0	11.3	20.1	0.031	24.6 (2)
15.0	30.0	11.3	40.1	0.042	26.3 (3)
11.3	15.0	15.0	10.9	0.031	21.0 (4)

Bichromate Solution A was used as the standard. The readings with Solution B were found to be inversely proportional to the bichromate concentrations at different settings. Readings 1 and 2 of the picramate solution were inversely proportional to the picramate concentrations with the same concentration and depth of bichromate solution. If proportionality to concentration held, Reading 3 of picramate solution should have been twice that of Reading 1 or 37.0, since the depth of the bichromate was twice as great. The value found was 26.3. From Readings 1 and 2, Reading 4 for picramate solution should have been 18.5. The value found was 21.0.

A number of series of results were obtained showing essentially the same relations as those given with greater or less variations depending upon the concentrations of bichromate and picramate. Since Benedict and Osterberg suggest the use of bichromate as

standard only if the other standards are not available, these results will not be given in detail. This difference in the color match is perceived much more readily by some eyes than by others. If the eye is very sensitive to such differences, the results will be correspondingly less accurate. This difference is also brought out clearly by inserting glasses of different colors, such as green, in the colorimeter so that the light before it reaches the eye passes through them. However, it is possible to obtain a match in most cases, but a greater latitude must be allowed here because of this difference.

The conclusions from the results may be stated as follows:

1. With a bichromate solution of unvarying concentration as a standard, always set at the same height in the colorimeter, solutions of picramate give readings inversely proportional to their concentrations.

2. Read against bichromate solutions of different dilutions set at the same height, or against the same bichromate solution set at different heights, picramate solutions do not give proportional readings.

The use of bichromate as standard depends, therefore, upon the determination of the colorimetric equivalence of bichromate and picramate for the definite colorimeter setting and concentration of the bichromate solution used.

Folin pointed out this same fact, in his paper on the determination of creatinine, using a bichromate solution as standard.[8] He has since abandoned bichromate in favor of creatinine itself.[9]

The differences pointed out apply in general to colorimetric comparisons of solutions of substances whose colors are visually alike but due to different absorption spectra. It was pointed out by Newcomer[10] that "It is thus essential in order that a color match be satisfactory under varying conditions that the absorption curves of the two substances composing the match be as near alike as possible." In any other case, the limitations of standard solution setting and concentration must be adhered to.

(*e*) *Picramate Plus Picrate against Picramate Plus Picrate (Varying Concentrations).*—Since the picrate sugar method in-

[8] Folin, O., *Z. physiol. Chem.*, 1904, xli, 223.

[9] Folin, O., *J. Biol. Chem.*, 1914, xvii, 469.

[10] Newcomer, H. S., *J. Biol. Chem.*, 1919, xxxvii, 486.

volves the use of two colored substances, it is of interest to compare the color values of these substances. An attempt was therefore made to determine the mg. of picrate equivalent colorimetrically to 1 mg. of picramate. A number of methods may be used based upon suitable colorimetric measurements.

When two solutions match (possess the same color value), the ratio of their color values per cc., or per mm. of height as determined in a colorimeter, is inversely proportional to these heights. Assuming proportionality of concentration to color value within a limited range, with one solution as standard, for the other solution the concentrations of picrate and picramate may be calculated which will give the same color value as the first solution. From these, the colorimetric ratio of picramate to picrate can readily be calculated.

The general relations involved may be expressed as follows:

P = Color value of 1 mg. of picramic acid (present as picramate).
p = " " " 1 " " picric " (" " picrate).
R_s = Reading of height of standard solution in colorimeter.
R_u = " " " " unknown " " "
s = Mg. of picramic acid (present as picramate) per cc. in standard solution.
s' = Mg. of picric acid (present as picrate) per cc. in standard solution.
u = Mg. of picramic acid (present as picramate) per cc. in unknown solution.
u' = Mg. of picric acid (present as picrate) per cc. in unknown solution.

For the same color value of the standard and unknown

$$sP + s'p = \frac{R_u}{R_s}(uP + u'p)$$

From this it follows that the ratio of the color values of the same weights of picramic and picric acids (present as their salts in alkaline solution) is given by the expression

$$\frac{P}{p} = \frac{u'\frac{R_u}{R_s} - s'}{s - u\frac{R_u}{R_s}}$$

Also, if the ratio $\frac{P}{p}$ is known, then, comparing two solutions both of which contain known concentrations of picrate, and one a standard amount of picramate, the concentration of picramate (calculated as picramic acid) in the unknown is given by the expression

$$u = \frac{R_s}{R_u}\left(s + s'\frac{p}{P}\right) - u'\frac{p}{P}$$

In the following experiments, the picrate and picramate are always expressed as mg. of picric and picramic acids per cc. of solution. Enough excess alkali is present in every case to bring out the necessary color as found in preceding sections.

In order to obtain the value of the color equivalence of picramate and picrate, series of experiments were carried out in which colorimetric comparisons were made between picramate on the one side and picramate plus picrate on the other; between picramate plus a small amount of picrate (having a color value very close to the solution recommended by Benedict) on the one side and picramate and picrate on the other; and between picramate and picrate on both sides. Table III shows some of the typical results obtained. Solutions A and B refer to the two being compared, and concentrations are given in the usual terms. The actual readings obtained are not given. These were varied over considerable ranges, but regularities connected with these variations were not observed. The color value ratios of picramate to picrate were calculated by means of the equation given in the table.

The first striking fact of these results is their apparent lack of constancy for the picramate-picrate ratio. This may be only apparent to some extent, as it was found in some cases that small differences in readings would cause comparatively large differences in the calculated ratios.

For example, a difference in the colorimeter readings of 0.3 mm. each would have resulted in the ratio of 1,000: 1 for the seventh, tenth, and thirty-second results where the ratios found were 750, 1,100, and 1,210 to 1. Similarly, a difference in the reading of 0.4 mm. for the first result and 0.6 for the fifth, where the found ratios were 440 and 810 to 1 would have given the ratio

TABLE III.

Solution A.		Solution B.		Color ratio picramate: picrate.
Concentration of picrate.	Concentration of picramate.	Concentration of picrate.	Concentration of picramate.	
mg. per cc.	*mg. per cc.*	*mg. per cc.*	*mg. per cc.*	
	0.080	7.2	0.080	440
	0.070	10.8	0.070	550
	0.060	14.4	0.060	650
	0.050	18.0	0.050	800
	0.040	21.6	0.040	810
	0.030	25.2	0.030	950
	0.035	5.4	0.035	750
	0.031	9.0	0.031	910
	0.027	12.6	0.027	950
	0.023	16.2	0.023	1,100
	0.019	19.8	0.019	980
	0.100	7.2	0.080	450
	0.100	10.8	0.070	590
	0.100	14.4	0.060	640
	0.100	18.0	0.050	780
	0.100	21.6	0.040	860
	0.100	25.2	0.030	840
	0.042	5.8	0.015	1,060
	0.042	5.8	0.018	1,060
	0.042	5.8	0.022	860
	0.042	5.8	0.025	910
	0.042	5.8	0.028	890
	0.042	5.8	0.032	790
(2.0)	0.042	5.8	0.015	1,140
(2.0)	0.042	5.8	0.018	1,070
(2.0)	0.042	5.8	0.022	1,130
(2.0)	0.042	5.8	0.025	910
(2.0)	0.042	5.8	0.028	890
(2.0)	0.042	5.8	0.032	1,080
(2.0)	0.042	5.8	0.036	1,190
(2.0)	0.042	5.8	0.040	1,390
(2.0)	0.042	5.8	0.044	1,210
(2.0)	0.042	5.8	0.048	990
(2.0)	0.042	11.5	0.015	1,340
(2.0)	0.042	11.5	0.018	1,270
(2.0)	0.042	11.5	0.022	1,320
(2.0)	0.042	11.5	0.025	1,180
(2.0)	0.042	11.5	0.028	1,220
(2.0)	0.042	11.5	0.032	1,120

TABLE III—*Concluded.*

Solution A.		Solution B.		Color ratio picramate: picrate.
Concentration of picrate.	Concentration of picramate.	Concentration of picrate.	Concentration of picramate.	
mg. per cc.	*mg. per cc.*	*mg. per cc.*	*mg. per cc.*	
(2.0)	0.042	11.5	0.036	1,010
(2.0)	0.042	11.5	0.040	950
(2.0)	0.042	11.5	0.044	910
(2.0)	0.042	11.5	0.048	810
5.8	0.032	5.8	0.016	790
5.8	0.048	5.8	0.016	1,040
5.8	0.064	5.8	0.016	920
5.8	0.048	5.8	0.032	1,310
5.8	0.064	5.8	0.032	1,180
5.8	0.064	5.8	0.048	1,220

of 1,000: 1. These results give perhaps the extreme variations for difference in ratio with small difference in colorimeter reading. The variation of the ratio is evidently connected with the differences in concentration of both picrate and picramate in both solutions. With four such variables to deal with, it is impossible to draw general conclusions with regard to the change with the results obtained.

The first twenty-three results were obtained by comparison with pure picramate solutions (containing excess of alkali). In the Benedict method, a standard solution containing picramate and picrate is prepared very nearly equivalent to the color developed by 1.0 mg. of glucose with the 4 cc. of picrate solution, heated for 10 minutes, finally diluted to 25 cc. Solution A with 0.042 mg. of picramic acid (as picramate) per cc. has a color value equivalent to that produced by this amount of glucose when the heating was continued for 1 hour. The next twenty experiments had for Solution A a color value equivalent to 0.042 mg. of picramic acid per cc. as before, but made up with the relative concentrations of picrate and picramate used by Benedict as standard. This contained very nearly 2.0 mg. of picric acid (as picrate) per cc., and a colorimetric equivalent less of the picramate. This is shown in the table by placing the 2.0 mg. of picric acid in parentheses.

Forty-nine results are given. Thirty-one of these give picramate-picrate color ratios between 800 and 1,200 to 1. Ten are below 800, of these two of 790 each, one of 780. Eight are above 1,200, of these one of 1,210, two of 1,220. The average of the forty-nine results gives a ratio of 963 to 1. The average of the thirty-one results (800 to 1,200) gives 984. Of the four results giving ratios less than 600 to 1, two were obtained with 0.080 mg. of picramic acid per cc., and two with 0.070 mg. of picramic acid per cc. Some experiments with greater concentrations of picramic acid gave still smaller ratios. These decreasing ratios with increasing picramate are evidently to be referred to the same cause as the lack of equivalence of bichromate and picramate solutions at different concentrations described in Section 4 *d*. The color values as determined by the eye vary differently in these solutions containing relatively more picramate than where small amounts are present.

In view of the possibility of experimental inaccuracy, and also because the ratio serves in the main as a correction term, the round value of 1,000 to 1 will be used here as the color value equivalent of picramic and picric acids (present as their salts). In solutions containing considerably larger concentrations of picramate than are met with in general work, this ratio will be less; if considerably less picramate is in solution than is found ordinarily, this ratio must be increased.

As stated before, this varying ratio of colorimetric equivalence of picramate and picrate probably is to be referred to the physical method of determining color values and not to any chemical change in the substances in solution. It is also probable that the ratio of the concentrations of picramate and picrate in the solutions is the predominating factor which determines their colorimetric equivalence, although, because of the measurements which were carried out, greater emphasis was placed upon the picramate concentrations given in the tables. That is to say, for high concentrations of picramate and low of picrate, 1 mg. of picramic acid (present as picramate) will possess a color value equivalent to less than 500 mg. of picric acid (present as picrate), possibly as low as 200 mg. in some cases. For low concentrations of picramate and high of picrate, 1 mg. of picramic acid (present as picramate) will be equivalent in color value to 1,500 mg. or

more of picric acid (present as picrate). For intermediate concentrations as shown in the table, the ratio is in the neighborhood of 1 to 1,000.

This lack of constancy in the equivalence of the color values of picramate and picrate obviously causes a measure of uncertainty in correcting for the presence of picrate in the colorimetric measurements. This is true especially when the solutions which are being compared contain widely different concentrations of the two constituents.

(*f*) *Glucose-Picrate Product against Picramate Plus Picrate (Varying Concentrations.*—In the preceding sections an attempt was made to describe the relations which appear to underlie the colorimetric comparisons of the glucose-picrate method. There will be presented in this section some of the results and conclusions obtained with glucose solutions bearing more directly upon the determination of glucose as described by Benedict and Osterberg.

Benedict and Osterberg used various solutions as their standards. In the first place they suggest a solution prepared from glucose at the same time and in the same way that the filtrates of the unknown solution are treated. Such a solution would contain 5.8 mg. of picric acid (as picrate) and colored substance obtained from 0.04 mg. of glucose per cc. The best permanent standard (giving a color value very nearly that given by the picrate solution with 0.04 mg. of glucose) as given by them contained very nearly 1.8 mg. of picric acid and 0.35 mg. of picramic acid (both present as their salts) per cc. Where pure picramic acid is not available, they suggested the use of potassium bichromate as a standard but indicated that the other standards are preferable.

Some points in their experimental procedure may be discussed briefly. Since they found that increasing the concentration of sodium carbonate in the glucose solutions produced continuously increasing color, the concentration recommended by them as best was adhered to strictly in this work. The length of time of heating the glucose solution to produce the colored substance is an important question. In order to obtain quantitative and comparable results in every case, it would appear that a definite reaction must take place leading to definite products. This implies that an end-point must be reached which is stable to a certain extent, so that further treatment within reasonable limits

will not produce further change. Benedict and Osterberg stated in their paper that 10 minutes heating sufficed, but in a private communication expressed the opinion that a longer time was advisable.

A number of series of experiments were carried out to test this view. Table IV gives some of these results. The solutions as read contained 0.040 mg. of glucose, 5.8 mg. of picric acid, and the usual concentration of alkali per cc. The procedure of Benedict and Osterberg was followed strictly, except for the different times of heating. Comparisons were made with the standard picramate solution recommended by Benedict and Osterberg.

TABLE IV.

Time of heating.	Colorimetric readings.		
	Solution I.	Solution II.	Solution III.
min.			
5	14.4	17.6	22.6
10	13.6	15.4	17.7
15	13.6	14.9	15.8
20	13.2	15.1	15.2
25	13.5	14.6	15.3
30		14.4	15.1
35		14.7	14.9
40		14.5	14.7
45		14.5	14.5
50		14.1	14.2
55		14.4	14.3
60		14.2	13.9

With Solutions I and II, the color was developed directly with the glucose solution; Solution III was submitted to the precipitation procedure with mercury salt and alkali before the color was developed.

With the salts from the precipitation procedure present, considerably longer time was needed for full color development. With pure glucose solution 10 to 20 minutes appear to be sufficient. There was practically no further change here on heating to 1 hour. Various factors influence the rate of production of colored substance from the picrate, such as alkalinity of solution, concentration of glucose, and the presence of other substances.

Benedict[11] emphasized the fact that very small amounts of acetic acid in excess lead to a remarkable decrease in the rate of color development, while Okey[12] pointed out the retarding effect of sodium chloride.

In view of these facts, and because it was considered inexpedient to heat for a short period of time on the assumption that the reaction had proceeded to a certain definite point, it was decided to heat all glucose solutions for 1 hour in studying this reaction. This length of time of heating results in a difference in the concentration of the permanent standard recommended by Benedict and Osterberg and prepared by color comparison with a solution from 0.04 mg. of glucose per cc. They found that a solution containing 1.8 mg. of picric acid and 0.035 mg. of picramic acid (present as their salts) gave a satisfactory standard for this amount of glucose. For the longer time of heating, and using the constituents in the solution in the same ratio that they did, it was found that the concentrations giving a color value equivalent to this amount of glucose contained very nearly 2.0 mg. of picric acid and 0.040 mg. of picramic acid (present as their salts) per cc.

The results obtained are given in Table V. The glucose solutions were treated with the sodium carbonate solutions recommended by Benedict and Osterberg, the volumes of glucose solution ranging from 1.0 to 4.0 cc. and the total glucose content from 0.42 to 1.5 mg., the concentrations given in the table representing mg. of glucose per cc. after dilution. These were heated for 1 hour, then diluted with water to 25 cc. or 12.5 cc., and compared with the standard solution of picrate plus picramate. The usual excess of alkali was present.

Columns 1 and 2 show the concentrations of picric acid and picramic acid (present as their salts) in the standard, Column 3 the concentration of picric acid (present as its salt) in the "unknown," and Column 4 the added concentration of glucose in the "unknown" solution. From the colorimetric readings, the concentration of picramate in the unknown was calculated, correction for the picrate present being made on the assumption that the colorimetric equivalents of the color values of picramate to picrate were in the ratio of 1,000 to 1. From these values and

[11] Benedict, S. R., *J. Biol. Chem.*, 1919, xxxvii, 503.
[12] Okey R., *J. Biol. Chem.*, 1919, xxxviii, 33.

TABLE V.

Standard.		Unknown.		Color ratio glucose: picramic.
Picric.	Picramic.	Picric.	Glucose.	
mg.	*mg.*	*mg.*	*mg.*	
5.8	0.016	5.8	0.034	1.44
5.8	0.016	5.8	0.038	1.38
5.8	0.016	5.8	0.040	1.36
5.8	0.016	5.8	0.042	1.29
5.8	0.016	5.8	0.054	1.26
5.8	0.032	5.8	0.034	1.36
5.8	0.032	5.8	0.038	1.31
5.8	0.032	5.8	0.040	1.29
5.8	0.032	5.8	0.042	1.26
5.8	0.032	5.8	0.054	1.19
5.8	0.048	5.8	0.034	1.38
5.8	0.048	5.8	0.038	1.32
5.8	0.048	5.8	0.040	1.28
5.8	0.048	5.8	0.042	1.29
5.8	0.048	5.8	0.054	1.20
5.8	0.064	5.8	0.034	1.35
5.8	0.064	5.8	0.038	1.30
5.8	0.064	5.8	0.040	1.28
5.8	0.064	5.8	0.042	1.23
5.8	0.064	5.8	0.054	1.17
5.8	0.020	5.8	0.034	1.33
5.8	0.020	5.8	0.038	1.31
5.8	0.020	5.8	0.040	1.32
5.8	0.020	5.8	0.042	1.30
5.8	0.020	5.8	0.054	1.20
5.8	0.040	5.8	0.034	1.26
5.8	0.040	5.8	0.038	1.32
5.8	0.040	5.8	0.040	1.26
5.8	0.040	5.8	0.042	1.20
5.8	0.040	5.8	0.054	1.17
Mean.....				1.287
5.8	0.016	11.5	0.034	2.06
5.8	0.020	11.5	0.034	1.75
5.8	0.032	11.5	0.034	1.92
5.8	0.040	11.5	0.034	1.84
5.8	0.048	11.5	0.034	1.80
5.8	0.064	11.5	0.034	1.79

TABLE V—*Concluded.*

Standard.		Unknown.		Color ratio glucose: picramic.
Picric.	Picramic.	Picric.	Glucose.	
mg.	*mg.*	*mg.*	*mg.*	
11.5	0	5.8	0.034	1.63
11.5	0	5.8	0.038	1.54
11.5	0	5.8	0.040	1.53
11.5	0	5.8	0.042	1.45
11.5	0	5.8	0.054	1.43
11.5	0.030	5.8	0.060	1.17
11.5	0.030	5.8	0.050	1.25
11.5	0.030	5.8	0.040	1.32
11.5	0	11.5	0.034	2.18
11.5	0.030	11.5	0.060	1.39
11.5	0.030	11.5	0.050	1.58
1.8	0.035	5.8	0.060	1.10
1.8	0.035	5.8	0.050	1.15
1.8	0.035	5.8	0.040	1.20
1.8	0.035	5.8	0.030	1.57
1.8	0.035	5.8	0.020	1.87
1.8	0.035	11.5	0.060	1.32
1.8	0.035	11.5	0.050	1.46
1.8	0.035	11.5	0.040	1.76
1.8	0.035	11.5	0.030	2.10
1.8	0.035	11.5	0.020	5.40

the concentrations of glucose given in Column 4, the ratios of the equivalents of color values of glucose to picramic acid (present as its salt) for equal weights of the two were calculated and are given in Column 5.

The striking fact observed with the glucose-picramic ratio is its lack of constancy. Two immediate assumptions are introduced in the calculations; namely, the value and constancy of the picramic-picric ratio, and the definite character of the reaction by which glucose is transformed into the colored substance. This reaction is assumed to lead solely to the formation of picramate. It is probable that while picramate is the main product of the reaction other substances, even if only in very small amounts, are produced as well.

With regard to the constancy of the picramate-picrate ratio assumed, any other constant values would give similar irregular results for the glucose-picramic ratio. Only by assuming different ratios for different amounts of picramic and picric acids could constant results be obtained. Such ratios would be dependent in part upon the concentrations of the two substances both in the standard and in the unknown solutions.

These general irregularities are due undoubtedly mainly to the optical properties of the solution and the errors inherent in the visual method of comparison described in the preceding sections. At the same time, it is possible that the times of heating and the concentration of the alkali during heating might influence the results.

The results may be treated somewhat differently. Two readings with different solutions may be combined in a pair of simultaneous equations, with the picrate-picramate ratio and the glucose-picrate ratio as the unknown quantities. Solving a number of these sets of equations which involve no assumption as to the color equivalence of picrate and picramate gave similar varying values for the two ratios, indicating that the variations are inherent in the nature of the results (or the experiments).

In spite of this lack of constancy, it may be possible to go somewhat farther with these results. The optical properties might be expected to cause the smallest interference if the solutions compared are most nearly alike in composition. This similarity is evidently obtained with the first thirty results in Table V. The mean value of the glucose-picramic ratio is 1.29 to 1 for these. This indicates a fairly definite chemical reaction, which it would be possible to formulate if more chemical evidence were available.

The remaining results given in the table show greater variations in the glucose-picramic ratio, but also contain much greater differences in the contents of the solutions being compared. As stated before, although it is possible that the chemical reaction leading to the production of colored substance by the action of glucose on picrate may be different depending upon the concentration of the glucose and the more or less accidental presence of other substances, it is more probable that this ratio is much more constant than the results would indicate and that the apparent inconstancy is caused by the inconstant picramic-picric ratio and the optical properties of the solutions being compared.

At the same time, it may be pointed out that the percentage of the total color which is due to the glucose is large. It may vary from 60 per cent for very dilute glucose solutions to 80 and even 90 per cent for the solutions met with ordinarily.

Benedict and Osterberg did not enter into these questions specifically in their paper. However, from the results of the permanent standard given by them, the ratio of the color values of glucose to picramic acid (present as picramate) is found to be 1.14 to 1. This ratio is less than that of the average of a number of the results given in Table V. It is uncorrected for picrate color and is based upon 10 minutes heating of the glucose solution.

It is evident that Benedict and Osterberg developed conditions involving concentration of picrate, concentration of alkali, time of heating, *etc.*, which permitted them to obtain results of a considerable degree of accuracy in the determination of glucose. The extension of their work, for which the results are presented here, indicates that the conditions which they realized are limited, especially with regard to the concentration of glucose which may be estimated accurately. Especially with very small amounts of reducing substances present, where the standard and unknown solutions differ considerably in content and concentration might less accurate results be expected.

Because of the factors which may be varied in the standard and in the unknown, no attempt will be made here to show the limits of accuracy of the determinations with different amounts of glucose.

CONCLUSIONS.

An attempt was made to outline some of the principles underlying the ordinary use of the colorimeter for quantitative work. In connection with colorimetric estimations in mixtures of two colored substances, the glucose-picrate colorimetric method developed in detail by Benedict and coworkers was studied. It was pointed out that the optical properties as determined visually with a colorimeter permitted of accurate estimations for such solutions only in special cases. In order to be certain of quantitative results, the standard and unknown solutions, containing two colored substances, should be very nearly alike in composition and concentration. The errors introduced otherwise may be

considerable. The experimental results leading to these conclusions are given in some detail for mixtures of picrate and picramate, and for mixtures of picrate and the reduction product of picrate obtained by the action of glucose.

It is desired to thank Dr. I. Greenwald for his invaluable aid in planning the experiments and in the treatment of the results. Thanks are also due Mr. R. P. Greene for continued help in the different parts of the investigation.

FAT-SOLUBLE VITAMINE.*

VI. THE EXTRACTABILITY OF THE FAT-SOLUBLE VITAMINE FROM CARROTS, ALFALFA, AND YELLOW CORN BY FAT SOLVENTS.

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During the course of the last few years, as fairly accurate quantitative data—that is quantitative in a biological sense—on the distribution of the fat-soluble vitamine in various plant materials had become available, it appeared increasingly desirable to attempt to concentrate this dietary essential for a study of its chemical properties. For some time we hesitated to initiate experiments of this nature as from our experience with the fat-soluble vitamine content of butter fat we were impressed with its instability under certain environmental conditions. Our attempts were therefore first directed to a study of its distribution (1, 2) and its stability under the conditions in which it is found in plant materials (3). While this work was far from being completed when these experiments were started our results were sufficiently inclusive to indicate that the fat-soluble vitamine as found in plants was very stable and that no immediate serious difficulty was to be anticipated even if no special precautions were taken to minimize its destruction.

The first experiments inaugurated were based upon our recently observed relations in the distribution of the fat-soluble vitamine in roots and maize with respect to the occurrence of certain yellow plant pigments (4). In the highly pigmented yellow roots such as yellow carrots and sweet potatoes there had

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been found an abundance of the vitamine as contrasted with the non-pigmented roots in which it was found absent or at most present in very small amounts. The same relations held with respect to maize. Out of four varieties of white maize extensively grown in the corn belt not a single one was found to contain appreciable amounts of the vitamine; yellow varieties of maize, on the other hand, never failed to give evidence of containing it in considerable quantities. It will be recalled that there are similar relations in the case of fats which have already been briefly touched upon (5), but which will be more extensively discussed in a later publication.

As the fat-soluble vitamine was originally discovered in butter fat (6, 7) the one property available for its extraction and its accumulation which naturally would suggest itself to us was its solubility in fats. But here it was very obvious that butter fat by reason of its prohibitive cost could not be used in extensive preparations as a source of this dietary essential. It was, however, hoped that plant materials rich in the vitamine might yield it to fats by appropriate treatment.

For experiments of this nature both green leafy materials and carrots were available. It was planned to saturate these with lard, olive oil, or corn oil, extract the fat with ether, and then incorporate the extract in a ration for the determination of the vitamine. Preliminary experiments with olive oil and alfalfa yielded us an extract containing chlorophyll which proved impotent as a source of the vitamine. As chlorophyll in itself obviously was a constituent not to be desired in the extract we gave up the use of alfalfa and turned our attention to carrots on which we used corn oil and ether as the solvents in one case and lard and ether in the other. Our results were entirely unsatisfactory; a lard-ether extract gave absolutely negative results and a corn oil-ether extract gave results indicating that only traces of the vitamine were contained in it.

We next turned our attention to the direct extraction of the vitamine by means of fat solvents. Desiring to avoid the contamination of our extracts with chlorophyll and the large amounts of waxes associated with the external surfaces of plant structures, we concluded to use carrots in these experiments. Moreover, we surmised that extracts of carrots by virtue of the palatability of

the roots would not prove distasteful to the animals. In this we were in error as the essential oils of the carrot in concentrated form are distinctly bitter. This proved a difficulty in our work, especially as our extracts were found so low in their content of the fat-soluble vitamine that it was found necessary to feed them in considerable concentration to obtain unequivocal demonstration of the presence of the vitamine. Ether gave entirely negative results, but alcohol, carbon disulfide, chloroform, and benzene gave fairly satisfactory evidence of solvent properties for the vitamine. In no case was the result entirely satisfactory as the extracted carrot residues when fed in small amounts as the sole source of the fat-soluble vitamine gave no evidence of having been seriously depleted in their vitamine content. It was evident to us that, while carrots contained the fat-soluble vitamine in abundant amounts, for some reason or other—probably on account of the presence of the colloidal pectins and hemicelluloses or possibly on account of the occurrence of the vitamine in molecular combinations—no satisfactory quantities of the vitamine could be accumulated from them. We then directed our attention to alfalfa again as our single failure to secure a potent fat extract with this material appeared no less promising than our results obtained with the same technique on carrots. We did not feel assured that fat solvents by themselves would not be efficacious in giving us active preparations, a surmisal which proved entirely justifiable as shown by our experimental results. Benzene and cold 95 per cent alcohol were found to be excellent solvents; ether gave far inferior results. With yellow maize, which was later added to the materials investigated, ether again gave poor results, but hot alcohol removed the vitamine almost quantitatively.

The apparent difficulties anticipated in connection with the production of a concentrated fat-soluble vitamine preparation now no longer appeared insurmountable. It remained to be seen whether our observations of the association in nature of plant pigments of the carotin and xanthophyll type, the so called carotinoids, would aid us in formulating additional experiments for the characterization of the vitamine. We had already found in numerous trials that the fat-soluble vitamine was not extracted by water which together with its demonstrated solubility in fat

solvents made it appear highly probable that we were dealing with a lipoidal molecule. As the carotinoids are obtained by ether extraction of a saponified alcoholic extract of leafy materials we applied such a procedure in an attempt to secure an active preparation from alfalfa. In this we were entirely successful. Furthermore, a fractionation of this material by the methods used to separate carotin from xanthophyll led to securing a crude carotin preparation which contained the fat-soluble vitamine in large amounts and a crude xanthophyll preparation which contained none or little of it.

EXPERIMENTAL.

For the demonstration of the presence of the fat-soluble vitamine in our extracts and extraction residues, rats were used as the experimental animals. The extracts fed in most cases—exceptions being noted in their proper places later—were evaporated on dextrin and as such incorporated in a basal ration of 18 per cent casein, 2 per cent agar, 3 to 5 per cent ether-extracted wheat embryo and 4 to 5 per cent salt mixture,[1] the remaining caloric requirements being furnished by dextrin. The animals were fed and watered daily and were weighed once a week. This has become the general routine procedure of the laboratory for practically all our experimental groups, some being weighed oftener, and has been found very satisfactory in that it offers excellent opportunity for the systematic observation of the changes in the condition of the animals with the progress of the experiment. The detailed notations which are the result of such observations have not been entered in the protocols as they are of no special interest to the reader. To us, however, they were indispensable as we could not always anticipate the final outcome of the experiments and therefore could not pass on the ultimate value of any sudden change in the animals' condition.

Solubility of the Fat-Soluble Vitamine of Carrots in Fats.

These experiments were carried out with lard and commercial corn oil, both of which contain no fat-soluble vitamine. 3,000 gm. of dried grated carrots were soaked with 500 gm. of the fat

[1] For composition of the salt mixture see Steenbock, H., and Gross, E. G., *J. Biol. Chem.*, 1919, xl, 505.

and allowed to stand 6 to 8 days at room temperature. They were then extracted with ether in the cold three successive times, most of the ether was removed *in vacuo*, and the final traces were dissipated by exposure to an air current. There remained 534 gm. of preparation in the case of the lard and 539 gm. in the case of the corn oil which represented extractives totaling 34 and 39 gm. respectively. Both preparations were of a deep orange color.

The lard preparation gave little indication of fat-soluble vitamine having been extracted. When fed at a 10.8 per cent level equivalent to 60 per cent of dried carrots none of the four rats in the experimental group, although they were started at 52 to 74 gm., attained a weight greater than 116 gm. All of them developed xerophthalmia in one or both eyes after 10 to 12 weeks. It is probable that initially the preparation was not so deficient in the vitamine as the above citation of conditions would lead one to believe. We infer this from the fact that chemical changes were taking place in the preparation, for although the preparation was kept as much as possible in the dark and in the refrigerator bleaching progressed rapidly; in 5 months all the orange-yellow color had been lost. It will be recalled that in butter fat we have observed a destruction of the fat-soluble vitamine simultaneous with the destruction of the pigment by exposure to heat (5), a phenomenon which we have since observed under other conditions as well. As butter owes its color to carotin, the same pigment found in our lard preparation, the inference that the preparation may originally have contained some demonstrable fat-soluble vitamine is not to be considered an impossible one.

With corn oil, considerable evidence that the fat-soluble vitamine was extracted by it is presented in Chart 1. In Lot 640 on corn oil alone, all the rats died within a period of 10 weeks and Rats 2556, 2557, and 2558 developed undoubted cases of xerophthalmia. With corn oil extract of carrots, on the other hand, when fed at the same level equivalent to 60 per cent of carrots none of the rats (Lot 532) died until they had been 24 weeks on the experimental ration and only two, Rats 2130 and 2129, developed xerophthalmia which were abortive attacks. It is true that on this extract the amount of growth that took place was negligible, but the mere fact that the animals maintained their body weight over a period of 24 weeks and recovered rapidly and

completely from incipient attacks of xerophthalmia, even though continued on the ration, can only be accounted for by the fact that some of the vitamine was present. Lot 625, fed on a ration containing fat-soluble vitamine added with butter fat, gives evidence that the presence of corn oil in a ration of itself will not prevent growth from taking place when all the dietary requirements

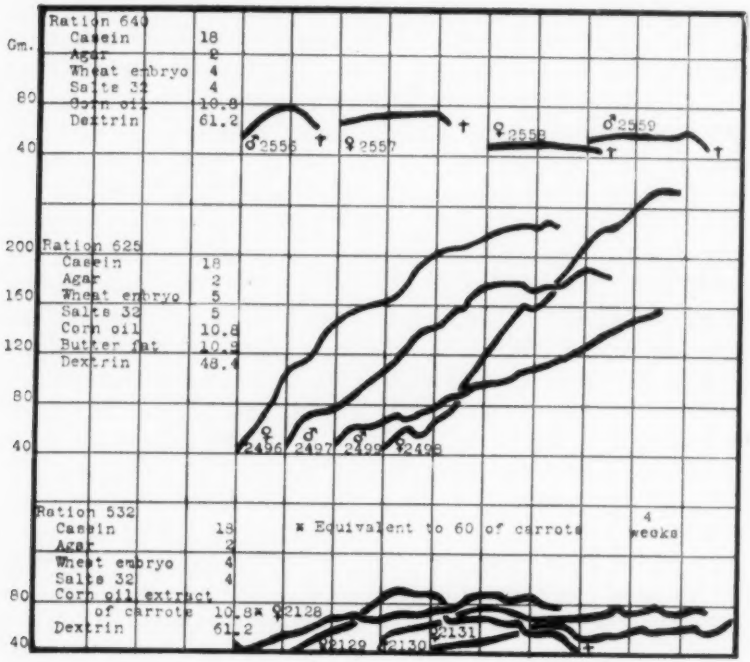

CHART 1.

of the animal are being met. In contrast with the lard preparation, the corn oil extract bleached very slowly; after 7 months it was still fairly highly colored, much more so than corn oil itself.

Extractability of the Fat-Soluble Vitamine from Carrots by Fat Solvents.

In the preparation of these extracts 6 kilos of the grated carrots, first air-dried and then dried over calcium chloride, were treated with the respective solvents at room temperature in large percolators.

The extractions were continued for three 24 hour periods and were so conducted that the first extract was used on a second portion of carrots and that on a third, each percolator receiving at least one treatment with fresh solvent. Except in the case of the alcohol extract the solvents were recovered by vacuum distillation. With the alcohol extract, foaming on concentration proved

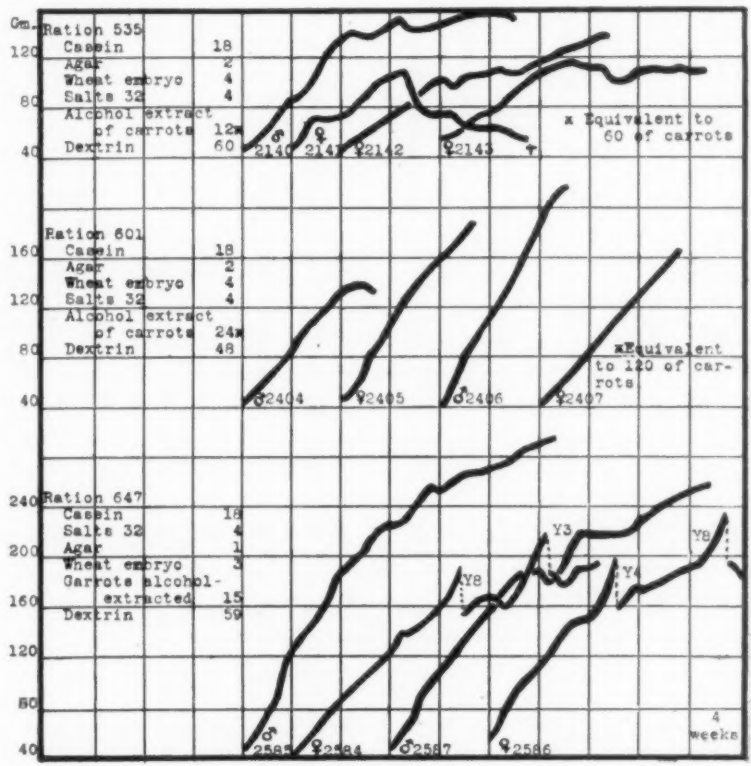

CHART 2.

so troublesome that it was found necessary to drive the alcohol off at room temperature with an air current. Ultimately all extracts were brought down on dextrin in an air current, the alcohol extract alone necessitating drying in a desiccator over calcium chloride as its high sugar content made it too hydroscopic for air drying.

TABLE I.

Preparation and amount of carrots or its equivalent fed.	Rat No.	Sex.	Weight of animals.						Notes on condition of animals.
			Initial.	4 wks.	8 wks.	12 wks.	16 wks.	20 wks.	
per cent			*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
Ether extract of carrots..... 60	2124	♂	52	84	87	55	Dead.		Rats 2124 and 2126 developed xerophthalmia in both eyes before death.
	2125	♀	45	82	88	Dead.			
	2126	♀	42	92	119	82	Dead.		
	2127	♀	44	73	88	Dead.			
Carrots, ether-extracted..... 15	2588	♂	52	110	159	204	224	231	At end of 25th week condition of animals varied from poor to good. Rat 2589 died during parturition. Rat 2591 raised one litter of four young and one of two young.
	2589	♀	49	105	139	179	Dead.		
	2590	♂	57	114	179	242	272	268	
	2591	♀	57	110	163	179	180	212	
Benzene extract of carrots..... 60	2136	♂	70	106	143	156	158	155	All animals were in good condition.
	2137	♀	68	96	122	131	137	145	
	2138	♀	53	85	100	117	136	148	
	2139	♂	53	97	119	145	161	172	
Carrots, benzene-extracted..... 15	2596	♀	50	95	150	179	199	195	At end of the 25th week all animals were in good condition.
	2597	♂	59	135	197	236	267	294	
	2598	♂	54	119	160	231	267	292	
	2599	♂	55	105	172	219	240	248	
Chloroform extract of carrots..... 60	2192	♂	41	57	80	116	163	171	At end of the 22nd week animals in fairly good condition but growth not satisfactory. Rat 2194 had one eye suggestive of incipient xerophthalmia.
	2193	♀	37	51	56	80	115	150	
	2194	♀	43	53	77	113	150	171	
	2195	♂	40	63	87	113	130	150	

Carrots, chloroform-extracted.....	15	2592	♂	57	114	162	216	250	265	When discontinued at end of 25th week condition of animals good. Rat 2593 raised one litter of six young beginning the 15th week.
		2593	♀	50	92	132	207	165	221	
		2594	♂	62	110	139	194	225	225	
		2595	♂	60	98	135	176	200	203	
Carbon disulfide extract of carrots...	15	2196	♀	35	40	55	65	72	80	10th week Rat 2196 had xerophthalmia in both eyes; later they improved. Rat 2199 had xerophthalmia in one eye in 11th week, in 13th week eyes were normal. Growth poor.
		2197	♀	35	45	54	58	46	Dead.	
		2198	♀	38	62	82	93	115	125	
		2199	♂	43	72	101	123	148	160	

All the extracts were fed at a level of intake equivalent to 60 per cent of carrots, and in a few cases equivalent to 120 per cent as well. In addition some of the extracted carrots were incorporated in an otherwise fat-soluble vitamine-free ration to determine the extent of their extraction.

In Chart 2, Lots 535, 601, and 647 represent graphically the results obtained with the alcohol extract and the extracted residue. As seen by the growth curves not until the extract was fed at such a level that the equivalent of 120 per cent of carrots was consumed was growth satisfactory. This indicates a very poor solubility of the vitamine in alcohol as carrots themselves fed at a 15 per cent level completely cover the animals' requirements. Further evidence that the carrots had not yielded any significant amounts of the vitamine to the alcohol is seen by the performance of the rats on the extracted carrots in Lot 647. They grew at an entirely normal rate and Rat 2586 even raised ten out of twelve young from two litters and maintained herself in excellent condition.

In Table I are presented data of the growth of rats on rations restricted in their fat-soluble vitamine content to that introduced with ether, benzene, chloroform, and carbon disulfide extracts of carrots, and the carrot residues. All the extracts were fed at such a level that an amount equivalent to 60 per cent of carrots was introduced in the ration. The extracted carrots were fed at a 15 per cent level.

By comparison of the weights of the animals receiving the extracts with those receiving the residues it is seen that none of the extracts was entirely satisfactory, ether giving the poorest results and chloroform and benzene the best. It appears significant that, while all the extracts were originally highly colored, the ether extract bleached very rapidly and after a few months, though kept in the dark and in the refrigerator when not in use, it had lost all color. The other extracts retained their color fairly well though a year later it was observed that the carbon disulfide preparation when set aside in diffuse light gradually lost its color in the course of a few weeks. Unfortunately the other extracts had all been used up and were consequently no longer available for this comparison, but it appears rather suggestive of the chemical nature of the fat-soluble vitamine that here again with the

destruction of the pigments there occurs a simultaneous disappearance of the vitamine. As in the case of the residue from the alcohol extraction, the ether, the benzene, and the chloroform extraction residues all gave little evidence of having lost large amounts of their vitamine. The carbon disulfide extraction residue was not fed.

The Extractability of Fat-Soluble Vitamine from Alfalfa.

With alfalfa, as with carrots, sufficient information on the fat-soluble vitamine content was available so that extractions could be carried out with a fair degree of assurance that although measurable quantities of vitamine were present the amounts were not so large that differences in content would not be readily perceptible. However, as a precautionary measure, to insure that a sufficiency of vitamine was present some of the material used was fed at a 5 per cent level in a fat-soluble, vitamine-free, but otherwise sufficient, ration. The alfalfa was a western grown sample available on the market as a meal for stock feeding purposes.

After its vitamine content was established, extractions were made with water, with ether, with benzene, and with alcohol, all extractions being conducted in the cold.

In the water extraction 1 kilo of alfalfa was soaked in a percolator with 6 liters of distilled water and gradually allowed to drain. As the water drained off more was added so that in the course of 2 days 8 to 10 liters of extract were accumulated. The alfalfa was then removed and dried before an electric fan. The extract was brought down on dextrin by the same means. The ether extract and the alcohol extract were made in a similar way except that in the case of the alcohol most of the solvent was distilled off in a water bath and recovered. This portion was also ultimately dried *in vacuo* as it proved to be very sticky. All extractions were continued for 48 hours with frequent renewal of solvent. In the preparation of the benzene extract 1,200 gm. of alfalfa were extracted with the solvent in three percolators, the extract of the one being used for the extraction of the second and that again for the third, a total of 7 kilos of solvent being used. At the end of the extraction most of the solvent was removed from the alfalfa by a powerful plant press and the remainder volatil-

TABLE II.

Preparation and amount of alfalfa or its equivalent fed.	Rat No.	Sex.	Weight of animals.						Notes on condition of animals.
			Initial.	4 wks.	8 wks.	12 wks.	16 wks.	20 wks.	
per cent			*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
	2680	♀	49	95	140	185	180	175	
Alfalfa, unextracted . . 5	2681	♂	47	102	177	215	215	230	Rat 2681 was rather thin. Other animals were in good condition.
	2682	♂	62	148	192	210	229	253	
	2683	♀	65	100	135	172	179	180	
	2552	♂	45	99	135	155	170	180	
Alfalfa, water-extracted 5	2553	♀	42	92	130	149	157	170	No xerophthalmia. Animals in poor to fair condition.
	2554	♂	43	97	138	150	200	225	
	2555	♀	61	95	136	134	160	167	
	2856	♂	69	165	120	Dead			
Water extract of alfalfa 20	2857	♀	56	122	Dead				Rats 2856 and 2857 developed xerophthalmia, the others did not.
	2858	♂	54	155	"				
	2859	♂	45	127	95	Dead			
	2568	♀	54	74	107	140	162	158	
Alfalfa, ether-extracted 5	2569	♂	64	93	165	185	137	Dead	Rats 2568 and 2571 looked rough; malnutrition of skin. Rat 2570 was blinded by xerophthalmia.
	2570	♀	58	78	104	141	149	100	
	2571	♂	60	140	174	192	242	232	
	2548	♂	45	92	152	205	245		
Ether extract of alfalfa 20	2549	♀	40	57	67	81	90		Condition of animals good except Rat 2549, which was very wild and nervous when discarded.
	2550	♀	45	89	120	145	169		
	2551	♂	52	82	122	139	132		

Alfalfa, benzene-extracted.....	5	2692	♂	64	147	195	225	197		All the rats suffered more or less from cutaneous malnutrition especially evident on the ears, nose, tail, and feet.
		2693	♀	45	87	117	93	Dead		
		2694	♂	52	69	90	111	119		
		2695	♀	48	62	72	101	98		
Benzene extract of alfalfa.....	5	2868	♂	55	142	184	199	200		All in good condition when discontinued. Rat 2871 raised two out of a litter of three young.
		2869	♀	62	109	135	180	210		
		2870	♂	75	140	168	197	185		
		2871	♀	52	102	142	147	155		
Alfalfa, alcohol-extracted.....	5	2684	♂	50	85	Dead				While none of the rats developed xerophthalmia, the eyes of all were either small or slightly swollen and red before death; suggested incipient attacks of xerophthalmia.
		2685	♀	52	115	"				
		2686	♂	64	110	77	Dead			
		2687	♀	47	45	50	"			
Alcohol extract of alfalfa.....	20	2948	♂	50	114	162	195	238	252	All in good condition.
		2949	♂	47	109	172	207	253	290	
		2950	♀	38	80	107	125	152	175	
		2951	♂	57	97	106	105	123	150	

ized at room temperature in an air current. The extract was brought down to a small volume *in vacuo*—after the addition of 5 gm. of calcium carbonate to neutralize the acidity—and then evaporated at room temperature on dextrin.

The results of feeding these extracts and their residues are brought out in Table II. The residues were fed at a 5 per cent level—as thereby any appreciable removal of vitamine would be revealed—and the extracts were fed at such a level that an intake equivalent to either 5 or 20 per cent of alfalfa in the ration was obtained.

Water, it will be noted, proved to be a very poor solvent for the vitamine as the rats dependent for their fat-soluble vitamine on a water extract of alfalfa died before 12 weeks of the experimental period had elapsed; moreover on the extracted residue they grew at a rate but slightly inferior to that observed on unextracted alfalfa. The slight discrepancy was no doubt occasioned by a partial destruction of the vitamine in the extractions as the early development of two cases of xerophthalmia in the group of animals on the extract makes it appear very improbable that it contained the vitamine.

Ether, U. S. P., freed from most of its water and alcohol over calcium chloride proved far more efficient than water in effecting a removal of the vitamine. On the alfalfa residue the rats were, however, still able to grow for a considerable time, but by the 20th week one rat had died and one had developed xerophthalmia. The extract fed at a level equivalent to 20 per cent of alfalfa proved fairly satisfactory.

Benzene gives indications of being a good solvent in view of the fact that the rats were able to grow well and even rear a few young on the extract fed at a level equivalent to 5 per cent of alfalfa. The residue also supported growth for some time, but when the experiment was discontinued the animals by the poor nutritive state of their skins and their decline in body weight gave evidence of an insufficiency of vitamine in their intake.

Alcohol, even when used in the cold, and without special precautions such as were used with benzene, appears to be an excellent solvent. This statement must be qualified as the extract was fed at a high level and therefore we have no assurance that smaller amounts would have been equally efficacious. This

would naturally follow if we were certain that no destruction of the vitamine had occurred in the process of extraction as the extraction residues were found so inactive. We are inclined to believe that the alcohol extract contained a large excess of the vitamine and therefore that very little destruction had occurred, as a similar extraction carried out with hot alcohol when distinctive processes would be accelerated gave us responses in growth of entirely equal magnitude.

Extractability of Fat-Soluble Vitamine From Maize.

Our observations on maize are limited to extraction with ether and alcohol. The data are to be considered as merely supplementary to those obtained with alfalfa, for while yellow maize contains considerable quantities of the fat-soluble vitamine it is questionable that we will ever use it for extensive preparations, as at the most its content of vitamine is only about one-seventeenth of that of alfalfa. In this work we used part of the same lot of Golden Glow maize of which the vitamine content was already known (4).

The ether extraction was carried out in a large Soxhlet extractor with U.S.P. ether freed from alcohol and water by calcium chloride. For the first 4 weeks the growth of the animals on the ether-extracted residue was so phenomenal, Chart 3, Lot 635, that it was decided to increase the extraction time—which had been 6 hours—to 18 hours. Even when fed on this more highly extracted maize, the rats continued to grow rapidly and maintained an excellent appearance. The feeding trials gave no evidence that any of the fat-soluble vitamine had been removed. The ether extract was not fed.

Our results with alcohol were of an entirely different order as alcohol proved to be an excellent solvent. At first we extracted the maize with cold alcohol using the completeness of removal of yellow pigment as the criterion for the progress of extraction of the fat-soluble vitamine. Cold alcohol extraction proved too time consuming so that later extractions were made at first with hot alcohol on the coarsely ground material in large Soxhlets and then followed by a cold alcohol extraction on the maize reground to a fine meal. It had been found that the hot alcohol

extraction could not be carried out on the finely ground maize as it invariably packed in the extractor to an impermeable mass. By the above mentioned course of procedure completeness of extraction was readily obtained, but we were not certain but that some destruction of the vitamine might have been brought

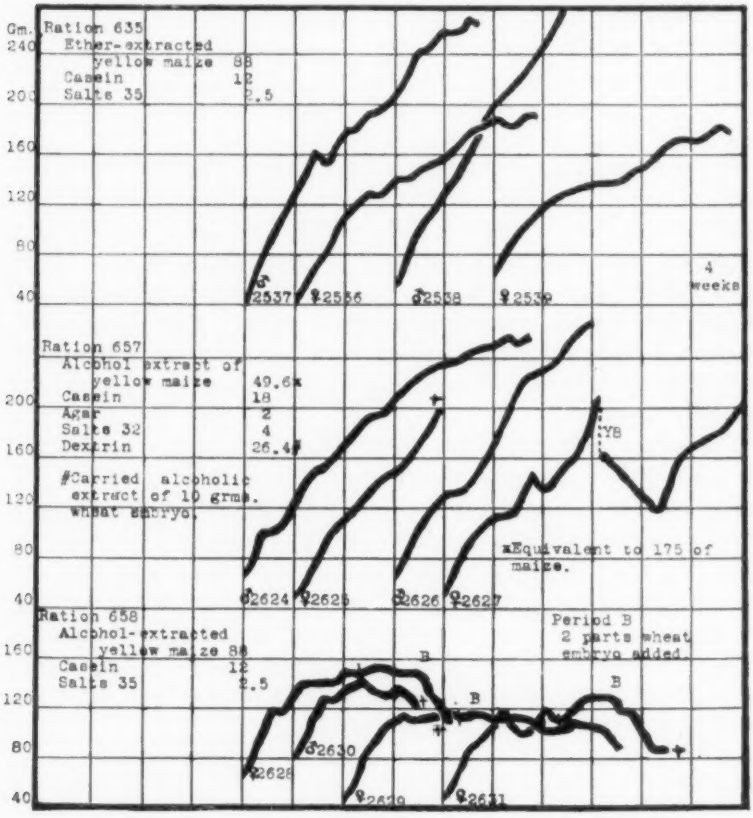

Chart 3.

about by the long continued boiling of the alcohol extracts. To minimize this, the hot alcohol extraction was interrupted at 6 hour intervals to enable the alcoholic solutions to be replaced with fresh alcohol. Three 6 hour periods were found sufficient for the extraction. The combined hot and cold extracts were

concentrated on the water bath, with recovery of the solvent, and finally were evaporated on dextrin for introduction into the rations.

The maize residues from the first cold alcohol extractions were fed to Lot 658, Chart 3. They were practically free from pigment, but here and there traces of yellow pigment were still discernable in some of the larger flint particles. As seen in the chart, even when these residues were suitably supplemented with protein and salts they did not cover the requirements of the animals as their growth was of but short duration. It was a bare possibility that the failure of growth to take place was partly due to a deficiency of water-soluble vitamine in the ration though earlier experiments of the laboratory had shown that the water-soluble vitamine is extracted from maize by alcohol with great difficulty. To eliminate this criticism the ration after 10 weeks was further supplemented with ether-extracted wheat embryo and as noted in the chart no improvement in the performance of the animals resulted. Rats 2628, 2629, and 2631 later developed xerophthalmia so that no reasonable doubt as to the deficiency of the ration in its fat-soluble vitamine content can be entertained.

In correlation with the above, and furthermore as evidence of the stability of the fat-soluble vitamine in the alcoholic extract, we have the entirely satisfactory growth of the animals in Lot 657, Chart 3. These rats received the alcoholic extract of maize equivalent to 175 per cent of the grain and therefore but little more than twice the amount of vitamine required by the animals as measured by the original content of maize equivalent. The excellent growth performance of the animals, together with the fact that Rat 2627 even raised two out of her litter of eight young, bears testimony to the fact that the fat-soluble vitamine is readily extracted from maize by alcohol and, furthermore, that it is fairly stable under these conditions.

Fractionation of Fat-Soluble Vitamine Extracts.

The extractability of the fat-soluble vitamine having been demonstrated, we next attempted to formulate a procedure which might give promise of separating the vitamine from contaminat-

ing substances in the extracts. From its occurrence in nature, it appeared plausible that methods designed for the isolation of pigments of the carotinoid type, such as carotin and xanthophyll, might possibly give a preparation rich in this dietary essential.

From the sum total of our experience on the extractability of the vitamine, benzene and alcohol appeared to be the most promising solvents and of these alcohol was selected because it is usually obtainable free from undesirable impurities and furthermore is readily removable from the extractives. As is well known, one of the methods employed in the separation of the carotinoids is the saponification of the pigment extracts which leaves them, together with other non-saponifiable substances, intact. Later these are then extracted from the saponified mixture with ether and subsequently crystallized from large volumes of solvent to the desired degree of purity. On these general experimental premises a number of preparations were made, but it has not yet been considered advisable to attempt fractionations by crystallization at least until more data on the stability of the fat-soluble vitamine are available.

In the first preparation the procedure was as follows: 3 kilos of alfalfa meal were extracted with cold alcohol in large percolators in the course of a few days. In these extractions the alfalfa was soaked with the alcohol and enough additional solvent poured on so that the material was well covered. After 24 hours extraction the percolator was drained, again filled up with solvent, extracted for 24 hours, and again drained. This was repeated once more and then, after washing the residue with a little additional alcohol, all extracts were united and distilled in a water bath down to a volume of about 2 liters. The concentrated extract obtained was of a dark green color, due to the large amount of chlorophyll extracted. For the saponification 225 cc. of 20 per cent alcoholic potassium were added and allowed to stand over night. This failed to complete the saponification so that it was found necessary to add 50 cc. and later another 25 cc. of the potash solution. This completed the saponification and the formation of the ether-insoluble potassium chlorophyllin in an additional 6 hours. The mixture was extracted repeatedly with ether till practically all yellow pigments had been removed, the ether solution then washed with water to remove alkali and salts,

and this in turn washed with ether. All ether extracts were then united, evaporated on the water bath to a small volume, and then again washed with a small amount of water. The solution thus obtained was of an orange-red color slightly tinged with brown but free from all green by reflected light. It was brought down on dextrin before an air blast for incorporation in a basal fat-

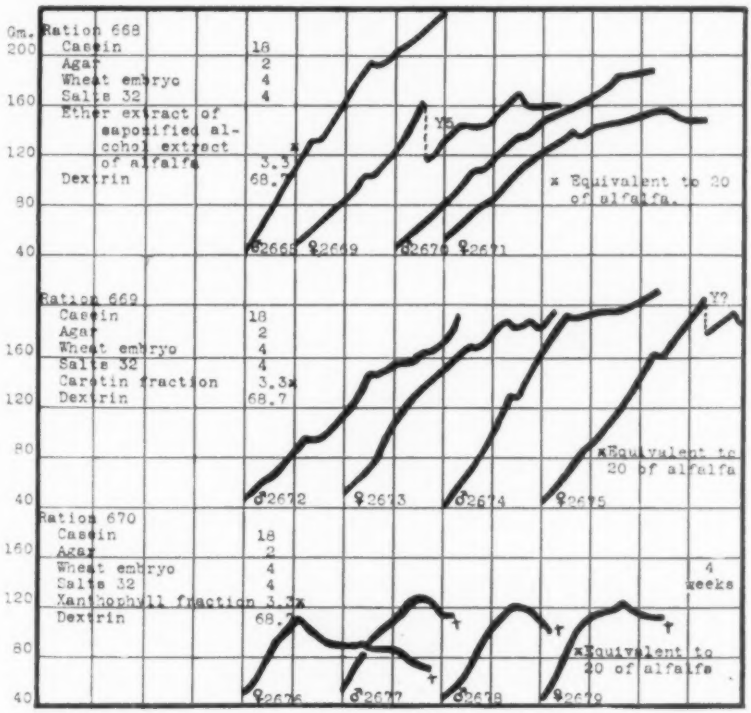

CHART 4.

soluble vitamine-free ration. The results of feeding this extract are shown graphically by Lot 668 in Chart 4. When the experiment was discontinued after having gone 22 weeks all the rats were in a fairly good condition. The evidence was indisputable; the vitamine had resisted the treatment to which it was subjected and finally had been successfully concentrated in ether solution.

Our next step was directed to determine whether the extract of the saponified alcohol extract of alfalfa could be successfully fractionated into an active and inactive fraction by a method such as has been used for the separation of the carotin and xanthophyll pigments. For this purpose 3 kilos of alfalfa meal were extracted as before with cold alcohol, the concentrated extracts were saponified with alcoholic potash, and then extracted with ether. Instead of evaporation on dextrin the ether extracts were freed from ether by exposure to an air blast. In the fractionation, the dark red residue was taken up in a mixture of alcohol and petroleum ether, transferred to a separatory funnel, and water added in small portions until the solution separated into two well defined layers—a lower one of diluted alcohol and an upper one of petroleum ether—both carrying considerable pigment. The respective layers were separated and reextracted—the alcohol fraction with petroleum ether and the petroleum ether with alcohol diluted with just enough water to bring about separation. Ultimately all alcohol solutions were united and all petroleum ether solutions were likewise united giving respectively the xanthophyll and carotin fractions. Each was evaporated on dextrin in an air blast and incorporated as the sole source of fat-soluble vitamine in an otherwise complete ration. The results from two such experimental groups are shown in Chart 4, Lots 669 and 670. They leave no doubt but what the carotin fraction contained an abundance of the vitamine while the xanthophyll fraction contained little or none of it. At the end of 17 weeks, when the feeding trials were discontinued, all the rats on the carotin preparation were in good condition, while all those on the xanthophyll fraction were dead, three having died before the end of the 10th week and one of these while suffering from a severe attack of xerophthalmia. The efficiency of this method of fractionation has been repeatedly demonstrated not only by different feeding trials, but also on different preparations. The presentation of these results together with detailed studies on stability, *etc.* will be reserved for later publications.

DISCUSSION.

In the light of the experimental data presented, there is abundant reason for assuming that attempts at isolation of the fat-soluble vitamine based on its solubility in fat solvents appear promising. As already stated, the difficulties of demonstrating its extractability were not so great as we had surmised when we were unaware of its stability under certain conditions and its lability under others. Furthermore we failed to anticipate the great variation with different substances in the ease with which the fat-soluble vitamine is yielded to certain fat solvents. It was no doubt the same premises that made McCollum and Simmonds (8) make the statement when speaking of the fat-soluble vitamine: "It is not extracted from plants with the fats by such solvents as ether, chloroform, benzene, or acetone, and is therefore not found in any fats or oils of plant origin. Hot alcohol does remove it from plant tissues." The reference to its solubility in alcohol is based on data obtained with the maize kernel which in themselves are not acceptable as McCollum, Simmonds, and Pitz (9) were of the opinion that maize generally was very deficient in the fat-soluble vitamine. Considering the evidence presented in a previous paper from this laboratory (4) such increased increments of growth as McCollum reported, due to the addition of an alcohol extract of maize to a basal maize ration, are entirely possible on the basal maize ration itself. Osborne and Mendel (10) also dissent from McCollum's point of view in regard to the non-extractability of the fat-soluble vitamine from leafy materials as they succeeded in obtaining a very potent oil from spinach and clover with U.S.P. ether. While our results with ether were not very successful we obtained abundant and unequivocal evidence that the fat-soluble vitamine can be extracted from plant materials with little loss of physiological potency with ether and with benzene, solvents which McCollum stated as being ineffective.

SUMMARY.

When carrots are saturated with lard or corn oil and then extracted with ether none or little of the fat-soluble vitamine is removed. The lard preparation gave no evidence of containing

the vitamine, but the corn oil preparation contained it in small but persistent amounts.

Ether has little solvent properties for the fat-soluble vitamine as found in carrots; chloroform and carbon disulfide remove some of it; while alcohol and benzene remove considerable amounts of it. None is entirely satisfactory for extraction purposes.

Fat solvents applied to alfalfa gave far more satisfactory results. Even on an ether extract one rat increased in weight 200 gm. during an experimental period of 16 weeks. Benzene and especially alcohol were found good solvents. Water was entirely ineffective.

While the vitamine is not extracted from maize by ether, alcohol removes it quantitatively and with little, if any, destruction.

The fat-soluble vitamine as extracted from alfalfa with alcohol resists saponification with alcoholic potash in the cold. From such a solution on dilution with water it can be extracted with ether. On fractionation by differential solubility there can be obtained a petroleum ether-soluble portion also containing carotin and a dilute alcohol-soluble portion which contains xanthophyll; the former contains the fat-soluble vitamine in large amounts, the latter little or none of it.

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A STUDY ON THE SEPARATION OF THE PHYSIOLOGICALLY ACTIVE PORTION OF THE POSTERIOR LOBE OF THE PITUITARY BODY.

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We are dealing in this paper with the uterus-contracting phase of the problem only. The basic material employed was a desiccated water extract, made by macerating and boiling finely minced posterior lobes with sufficient distilled water containing 0.25 per cent of glacial acetic acid to make the final volume represent 10 per cent of the fresh gland. On desiccation the liquid yielded about 0.7 per cent of total solids in the form of light yellowish hygroscopic scales. Several batches were thus made and combined into a uniform sample which represented the glands from 5,000 cattle. The chemical analysis of this extract did not reveal any striking peculiarities. The ash was mostly water-soluble, of alkaline reaction, and yielded CO_2 on addition of acid.

Ether, chloroform, petroleum ether, and methyl alcohol were ruled out as solvents for the active principle since the first three did not yield therapeutically active preparations and methyl alcohol extracted more impurities and less of the active principle than did ethyl alcohol. It was definitely established that the physiologically active portion was practically insoluble in absolute ethyl alcohol, very sparingly soluble in cold 95 per cent alcohol, but quite freely extracted with boiling 95 per cent alcohol.

10 gm. portions of the dried water extract, powdered over a 100 mesh sieve were extracted ten times with 50 cc. portions of 95 per cent ethyl alcohol at 20°C., and then eight times with equal volumes of boiling alcohol.

The combined cold alcohol extracts were evaporated until crystals appeared. After complete crystallization they were

filtered, washed with alcohol and ether, and found to constitute 3 per cent of the dried basic water extract. On recrystallization from water and analysis they were found to consist of approximately 67 per cent potassium chloride and 33 per cent sodium chloride. If the crystals are not separated at this stage they will appear in the dried residue and are then removed with difficulty. During the earliest experimentation where only small amounts of extract were available, these crystals escaped detection until the alcoholic extracts were evaporated to paste consistency. They could then readily be observed with the naked eye. The microscope revealed beautiful large cubes and fern leaf formations embedded in the yellow paste. The remaining alcohol extract, after removal from the crystals, was evaporated to dryness, a small portion at 50°C. and the balance at about 70°C. The total yield was 23.4 per cent of the dried basic material. The extracts were yellowish brown in color, exceedingly hygroscopic, and possessed a characteristic pungent odor. Analysis showed this fraction to be rich in water-soluble mineral matter and organic extractives of a non-protein nature.

The hot alcohol extracts filtered clear when boiling hot, but yielded a white amorphous fraction on the slightest cooling, while the active substance remained in solution throughout. If the active principle were sparingly soluble in cold and freely soluble in hot alcohol, it would naturally be precipitated with the inert material on cooling, but such is not the case. We are apparently here confronted with some form of hydrolysis whereby a protein complex is split by alcohol. The amorphous precipitate amounted to only 1.2 per cent of the dried basic material and was too small for quantitative analysis. It was of a protein nature, soluble in water, and physiologically inert. The filtrate from this amorphous fraction was evaporated to dryness, one part below 50°C., and another at about 70°C. The total yield was 14.6 per cent of the dried basic material. This preparation was yellowish in color, very hygroscopic, and readily soluble in water. It contained a large amount of organic and inorganic salts and the total nitrogen was higher than that of the cold alcohol fraction, but not equal to that of the basic water extract.

The alcohol-insoluble residue was dried, and averaged 57.8 per cent of the dried basic material. It formed a cream-colored

powder, much less hygroscopic than the alcohol extracts, and only partly soluble in water. The ash consisted principally of water-insoluble phosphates. The total sum of moisture, ash, and N × 6.25 approaches 100 per cent, indicating that this fraction is principally protein.

The physiological activity of all preparations was determined by the isolated uterine strip method. Samples of the original water extract before desiccation were ampuled, sterilized, and used as control. It will be seen from Table I that while the activity of the water extract was not affected by desiccation at 50–70°C. that of the alcohol extracts was markedly changed. The hot alcohol extract, which was dried below 50°C., was about 3.5 times stronger than the control, while the one dried at 70°C. was only 1.5 times as strong, plainly indicating the extraordinary sensitiveness of the active substance in this form to heat during drying. An independent experiment brought to light the fact that prolonged boiling of the dried water extract with alcohol, under reflux condenser, and evaporation of the extract at 100°C. rendered the preparation inert. We also found that while the hot alcohol extracts were exceedingly sensitive to heat on drying they retained their activity quite well in water solution and could be autoclaved without material loss in physiological value. The cold alcohol extract was only one-seventh and the alcohol-insoluble residue about one-fifth the strength of the control. The distribution of the physiological activity in the various fractions is as follows:

	per cent
Cold alcohol extract	3.5
Hot " "	51.0
Alcohol-extracted residue	11.5
Loss in process (by difference)	34.0

Most, if not all, of the loss in physiological activity in our present method of separating the active portion evidently occurred during the desiccation process of the hot alcohol fraction.

Table I shows that the hot alcohol extract, the most active physiologically, responded only slightly to the biuret test and to protein and alkaloidal precipitants. Experiments were carried out with uranium acetate, phosphotungstic acid, and tannic acid

TABLE I.

	Original 10 per cent acidulated water extract of the fresh posterior lobe.	Same extract desiccated.	Cold ethyl alcohol extract.	Crystals formed during evaporation of cold ethyl alcohol extract.	Hot ethyl alcohol extract.	Precipitate formed during cooling of hot alcohol extract.	Alcohol-extracted residue.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Yield		100.0	23.4	3.0	14.6	1.2	57.8
Moisture	99.3	6.95	1.75		3.32		3.65
Total nitrogen		11.16	7.25		9.87		13.94
Ash		13.87	21.30	100.0	16.38		7.14
Cl		1.93	1.73	51.95	1.75		Negative.
P		1.00	0.50	Negative.	0.70		1.44
K		6.56	Present.	34.79	Present.		Negative.
Na		0.93	"	13.26	"		"
Reaction to litmus of water extract of ash.		Alkaline.	Alkaline.	Neutral.	Alkaline.		Neutral.
CO_2 in ash		Present.	Present.	Negative.	Present.		Negative.
Physiological activity compared with original water extract; desiccation at 50°C.	Control.	Equal to control.	15 per cent of control.	Inert.	3.5 times stronger than control.	Inert.	20 per cent of control.
Physiological activity; desiccation at 70°C.	"	" "	" "	"	1.5 times stronger than control.	"	" "

Biuret reaction.....	Strong.	Strong.	Doubtful.		Positive.	Very strong.	Strong.
Tannic acid.....	Heavy precipitate.	Heavy precipitate.	Slight precipitate.		Precipitate.	Heavy precipitate.	Heavy precipitate.
Phosphotungstic acid..	" "	" "	" "		"	" "	" "
Potassium mercuric iodide.....	Precipitate.	Precipitate.	" "		Slight precipitate.	Precipitate.	" "
Picric acid.....	"	"	Turbidity.		Heavy cloud.	"	" "
Bromine water.....	"	"	"		Slight precipitate.	"	" "

on freshly prepared acidulated water extracts of the gland. The resulting filtrates and precipitates were freed from respective reagents in the appropriate manner. In each case the preparations from both filtrate and precipitate possessed sufficient uterus-contracting power to render this method of separating the physiologically active portion of the gland incomplete. This condition further indicates split products. When picric acid in alcohol was added to a water extract of the fresh gland a precipitate, partly flocculent and partly crystalline, was formed on standing. The crystals were found to be identical with the inert salts described above.

SUMMARY.

Evidence is furnished which indicates that the uterus-contracting active principle of the posterior lobe of the pituitary body does not occur in the fresh gland in free or crystalline form, but is linked to or part of some protein complex.

In its original protein association the uterine stimulus is insoluble in ether, petroleum ether, chloroform, and practically insoluble in absolute alcohol. It is sparingly soluble in 95 per cent alcohol, but yields a highly active split product when treated with hot 95 per cent alcohol. The split product is amorphous in nature, very hygroscopic, and much more sensitive to desiccation than the original basic material from which it was derived.

The crystalline bodies naturally occurring in and isolated from the posterior lobe are inorganic salts possessing no uterus-contracting power.

Alkaloidal reagents only incompletely precipitate the active fraction of the water extract of the gland.

The work is being continued.

EXPERIMENTS ON THE UTILIZATION OF SALEP MANNAN.

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In 1911 the author[1] reviewed the literature on the utilization of various polysaccharides from plant sources and reported some experiments on the utilization of mannan derived from the tubers of *Orchis* and *Eulophia*, popularly known as salep. It was then shown that this anhydride of mannose, common in the dietary of certain peoples in eastern Europe, was not hydrolyzable by enzymes of saliva, malt diastase, and pancreatic and intestinal juice, but that in feeding experiments it disappeared almost completely from the alimentary tract in man, the coefficients of digestibility in four experiments on two subjects being in three cases 100 per cent and in the fourth 94 per cent. Digestion of salep mannan with fecal bacteria resulted in the production of appreciable amounts of sugar, as has since been shown by Okey [2] to be the case with inulin.

There has never been any convincing evidence that carbohydrates resistant to the normal action of body enzymes can play a significant rôle in human nutrition, though attempts have been made to demonstrate a rise in the respiratory quotient after their administration, both in the case of inulin [3] and of agar-agar.[4] The respiratory quotient after administration of 200 gm. of

[1] Swartz, M. D., *Trans. Connecticut Acad. Arts and Sc.*, 1911, xvi, 247.
[2] Okey, R., *J. Biol. Chem.*, 1919, xxxix, 149.
[3] Goudberg, A., *Z. exp. Path. u. Therap.*, 1913, xiii, 310.
[4] Lohrisch, H., *Z. exp. Path. u. Therap.*, 1908–09, v, 478.

inulin reached a maximum of only 0.89 in the 3rd hour after ingestion as compared with a rise to 0.92 in $2\frac{1}{2}$ hours after feeding 150 gm. of oatmeal; and the quotient for soluble agar (100 to 110 gm.) reached a maximum of 0.86 in the 4th hour, the values in the preceding and succeeding hours being little if any above the fasting level. It does not seem therefore that sugar produced by fecal bacteria from such carbohydrates as inulin is available in any considerable amount for the host. Nevertheless, it appeared worth while to test this point in regard to salep mannan as it had been tested for inulin. The experiments herein reported were carried out before 1915, when they were interrupted because the supply of salep was cut off by the war.

EXPERIMENTAL.

Coefficient of Digestibility.

In the course of this work determinations of the coefficient of digestibility were made on several more subjects, with larger quantities of material than had been hitherto used. Two healthy young women, each weighing 70 kilos, consumed in 3 days a total of 75 gm. of salep mannan as a part of a simple cellulose-free ration. The experimental period was preceded and followed by 4 day periods in which the same diet was used, save that tapioca took the place of salep. The mannan was extracted from the tubers with water; the starch present digested off; the mannan precipitated by alcohol, so as to remove the sugar from the starch digestion; dissolved and reprecipitated; washed with ether, and dried. It was then in the form of a convenient white powder, dissolving readily in water. On account of its gelatinizing quality, the ingestion of any considerable amount was difficult, and led to the use of the dry powder in capsules in subsequent human feeding experiments. The glucose equivalent of the salep eaten was 61 gm., and the total excess of carbohydrate in feces during the salep period as compared with the fore and after periods was 1.7 gm. (Subject M) and 2.8 gm. (Subject H) reckoned as glucose, giving coefficients of digestibility for the mannan of 97 per cent and 95 per cent respectively (Table I).

TABLE I.

Determination of Coefficients of Digestibility.

	Subject M.	Subject H.
	gm.	*gm.*
Total carbohydrate intake per day	315	315
Carbohydrate in feces per day, fore period	0.53	0.50
" " " " " after "	0.56	0.47
Average output per day, fore and after period	0.55	0.47
" " " " salep period	1.09	1.42
" excess, salep period over fore and after periods	0.55	0.94
Total excess, salep period (3 days)	1.65	2.81
Dextrose equivalent to salep eaten	61.15	61.15
Coefficient of digestibility for total carbohydrate, salep period, *per cent*	99.6	99.5
Coefficient of digestibility of salep mannan, *per cent*	97.3	95.4

TABLE II.

Summary of Coefficients of Digestibility of Salep Mannan in Human Feeding Experiments.

Subject.	Weight.	Length of salep period.	Salep fed per day.	Glucose equivalent of salep fed.	Coefficient of digestibility.
	kg.	*days*	*gm.*	*gm.*	*per cent*
Z (Woman)*	52	2	30†	22.7	100
X " *	42	2	30†	22.7	94
Z " *	52	2	20	18.0	100
P " *	56	2	20	18.0	100
M "	70	3	25	20.5	97
H "	70	3	25	20.5	95
R (Man)		1	45	20.5	97
B (Boy)	33	3	56	51.8	96
Average					97

* Swartz,[1] pp. 354, 355.

† Salep powder corresponding to 22.7 gm. of glucose.

In another case a diabetic man under supervision in a hospital was given in a single day 45 gm. of salep mannan in capsules, the diet otherwise being only broth, black coffee, and 2 ounces of whiskey. The feces were marked for the day by carmine and a dose of 1 ounce of magnesium sulfate was given with the morning

broth. There was no inconvenience from gas formation as with inulin, and only 2.7 per cent of the carbohydrate fed was recovered in the feces, making the coefficient of digestibility in this case 97 per cent.

In a third case a 15 year old diabetic boy weighing 33 kilos served as subject in a series of experiments to determine the fate of salep in the diabetic organism. As a part of this work a 3 day digestion experiment was conducted, with a cellulose-free basal ration, to which was added salep mannan to the amount of 33, 65, and 70 gm. on 3 successive days. There was no discomfort, even from the 70 gm., and the coefficient of digestibility for the period was 96 per cent.

Altogether, the average of eight experiments on seven human subjects is 97 per cent, the range being from 94 to 100 per cent (Table II).

Influence on Nitrogen Output.

In the experiment on the two young women cited above, a careful study of the nitrogen balance was made. It was hoped in the beginning that any failure to utilize the salep might be

TABLE III.

	Period No.	Time.	Nitrogen in feces per day.	Weight of air-dry feces per day.
		days	*gm.*	*gm.*
Subject M.	1	4	0.64	14
	2	3	1.00	21
	3	4	0.65	15
Subject H.	1	4	0.59	13
	2	3	1.44	27
	3	4	0.64	14

detected by a change in the nitrogen output if the diets were planned just barely to meet the energy requirement, and a food known to be readily digested—tapioca—was used in the fore and after periods, but replaced by an equivalent amount of salep in the mid period. The amount of salep which the subjects found it possible to take was not sufficient, however, to exert any marked influence. Hence the most striking result of the study of the nitrogen balance was the increase in the fecal nitrogen during the salep

period, amounting to 54 per cent in one case and 135 per cent in the other. There was also an increase in the volume of the dry feces, amounting to 46 per cent in one case and 105 per cent in the other. The amount of nitrogen and weight of air-dry feces per day are shown in Table III.

There was a slight fall in the urinary nitrogen in the salep period, but there were increases of 16 and 17 per cent respectively in the after period. This was probably due to a decided decrease in the urine volume during the salep period, and a subsequent "flushing out," the salep being very hygroscopic.

Fate in the Diabetic Organism.

Several attempts were made to discover whether salep could be converted into sugar in the diabetic organism. In the case of the diabetic man cited above, there was no sugar in the urine on the day of salep feeding, although the subject had been excreting sugar whenever carbohydrate was fed in ordinary forms. This same man on a previous day had received 10 gm. of salep without the appearance of sugar. The 15 year old diabetic boy was under observation for some months, the plan being to give salep in periods alternating with a salep-free but controlled diet, and to study the output of sugar, ammonia, and β-hydroxybutyric acid in the urine. The patient was not in a hospital and it was found toward the close of the work that he had not always followed instructions, even though his diet was administered in the laboratory. The most that can be said, therefore, with regard to sugar, was that there was no marked increase in any of the salep periods. In one of the best controlled periods lasting 5 days, salep was given averaging 56 gm. per day, but did not stop the production of β-hydroxybutyric acid which rose from 3.4 gm. on the day preceding the salep feeding to 15 gm. by the 2nd day, continuing about the same the 3rd day, and being promptly reduced by sodium bicarbonate administered in connection with green diet containing no salep on the 4th day. Both acetoacetic acid and ammonia showed slight increases during this period. While this work needs to be repeated if it is possible to get supplies of salep again, there was no evidence of salep being converted into sugar in the diabetic organism.

Glycogen Formation in Rabbits.

It was thought that in an herbivorous animal like the rabbit glycogen storage might possibly be demonstrated for this carbohydrate in the absence of specific body enzmes for its hydrolysis, through the activities of sugar-producing bacteria, as mannose has been shown to form glycogen readily.[5] Accordingly a

TABLE IV.

Glycogen Storage in Rabbits.

Subject.	Initial weight.	Time fasted.	Time fed.	Material fed.	Amount fed per day.	Weight of liver.	Glycogen recovered.	Ratio of glycogen recovered to weight of liver.
	gm.	*days*	*days*		*gm.*	*gm.*	*mg.*	*per cent*
I (Control)	1,275	6				18	35	0.20
II "	1,522	5	1	Starch. Lactose.	10 5	32	209	0.65
III*	1,262	6	1	Salep.	30	28	35	0.13
IV	1,495	6	1	"	15	22	5	0.02
V†	1,700	5	3	"	12.5	29	22	0.08
VI‡	1,418	5	3	"	20	28	16	0.06

* The contents of the stomach and intestines were collected and hydrolyzed, and carbohydrate was determined as glucose. 7 gm. were obtained from the large intestine, 1.5 from the small, and 1.5 from the stomach, or a total of 10 gm. That this sugar was largely, if not wholly, mannose was shown from a rich yield of mannose hydrazones in the hydrolyzed solution.

† Stomach and intestines yielded 5.9 gm. of glucose.

‡ In this case the fermentation was very marked. The contents of stomach and intestines were removed and 4.8 gm. of the mannan isolated and identified.

number of experiments were conducted to test this point. The animals were fasted from 5 to 6 days and then fed salep mannan in solution by means of a stomach sound, for from 1 to 3 days. They were killed 12 to 15 hours after the last feeding and the livers immediately removed and analyzed for glycogen, according to Pflüger's method.[6] Control rabbits were fed by the same

[5] Neuberg, C., and Mayer, P., *Z. physiol. Chem.*, 1902-03, xxxvii, 530.

[6] Pflüger, E. F. W., Das Glykogen und seine Beziehungen zur Zuckerkrankheit, Bonn, 1905.

method equivalent amounts of soluble starch alone, or of soluble starch and lactose together, the former proving preferable as the lactose induced diarrhea. The results of these experiments are summarized in Table IV. Two of the controls suffered accidents, but inasmuch as the storage of glycogen in the rabbit has been extensively studied, and the results with the salep are so definite as compared not only with the controls given in the table but with other work of this sort, it is felt that this loss was not serious. The rabbit fed only 15 gm. of the starch-lactose mixture showed a storage of 209 mg. of glycogen, while the storage in the salep rabbits resembles that of the fasted control, and in fact never reaches so high a percentage of the total weight of the liver. The four cases of salep administration gave practically starvation values. This is in sharp contrast to the results of feeding mannose as reported by Neuberg and Mayer [5] who found that from 8 to 10 gm. of mannose similarly administered yielded such values as 0.973, 0.606, and 1.01 gm. of glycogen. Salep seems to be even less capable of glycogen formation in the rabbit than is inulin.[7]

SUMMARY.

Salep mannan disappears almost completely from the human alimentary tract when fed in amounts varying from 20 to 56 gm. per day (corresponding to 18 to 52 gm. of glucose), as shown in eight feeding experiments in which the coefficients of digestibility range from 94 to 100 per cent, averaging 97 per cent. Four of these are reported in this paper.

The total nitrogen of the feces and the total volume of dry feces are increased by salep feeding.

Salep feeding did not result in glycosuria in one diabetic studied, nor did it arrest the production of β-hydroxybutyric acid in another.

Although rabbits readily store glycogen when mannose is fed, the administration of salep mannan resulted in no glycogen in rabbit livers above ordinary starvation values.

[7] Nakaseko, R., *Am. J. Physiol.*, 1900-01, iv, 246.

Salep resembles inulin in the hydrolysis by fecal bacteria, and in the readiness with which it disappears from the alimentary tract, but it does not form gas so readily. There was no evidence of gas or diarrhea except in one rabbit. It is less capable of glycogen formation in the rabbit than is inulin.

AT WHAT LEVEL DO THE PROTEINS OF MILK BECOME EFFECTIVE SUPPLEMENTS TO THE PROTEINS OF A CEREAL GRAIN?*

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Definite information on the quantity of milk proteins necessary to supplement with maximum efficiency for growth the proteins of a cereal grain is not available. The question is a decidedly practical one. Advice as to how much milk to be used daily per individual in the human dietary is now freely given—with recommendations varying from 1 pint to 1 quart—but on what scientific evidence such advice rests is not apparent. Experiments with growing swine on the proportion of skim milk necessary to supplement corn-meal (maize) have been conducted by Henry.[1] His results indicated that with one to three parts of skim milk to one part of corn-meal satisfactory growth could be made. These experiments were never longer than 10 weeks.

Manifestly the quantity of liquid whole milk to use as a source of vitamines or salts needed for normal growth will depend on the nature of the rest of the dietary. It is apparent, therefore, that a quantitative expression of the amount of milk needed daily may have little significance as far as vitamines and salts are concerned. This applies particularly to the mixed dietary of children. The situation may receive safer treatment if more importance is attached to the value of the proteins of milk and the basis for milk intake related to their supplementing power. Of course,

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

[1] Henry, W. A., *Rep. Wisconsin Exp. Station*, 1895, **7**.

it is true that there may occur composite diets of plant origin, whose protein mixture may be of fair efficiency at low planes of intake; further, it is also possible for meat proteins to supplement effectively the plant proteins, thereby lessening the quantity of milk needed where meat is part of the diet; but as far as we are aware there is no mixture of proteins—with the possible exception of egg proteins—when used either alone or as a supplement to the cereal grain proteins—that is equal in efficiency at as low a level as that of milk, which gives to the latter a unique place among foods.

There are two methods of experimental inquiry open for investigating this question. One is to measure over comparatively short periods of time the nitrogen retention of a growing animal on a protein mixture made from a cereal grain and milk; the other is to determine the rates of growth over longer periods of time of animals fed rations with varying proportions of cereal and milk proteins. We have used both methods; the first with growing swine and the latter with growing rats. The results of the experiments with the latter will be embodied in a later publication.

In our work on nitrogen retention with growing swine the method of procedure has already been described.[2] We chose to keep the cereal proteins used constant and allow the proportion of milk proteins to vary. With increasing levels of milk protein intake this procedure modified slightly the total plane of protein intake. In the several rations used the level of protein ingested varied from 8.6 to 10.9 per cent of the ration. The effect on the final conclusions would be to give slightly higher values of efficiency to the lower planes of protein intake. This affected in no way the interpretation of the data which can be seen in Chart 1. In this chart, for purposes of clarity, the corn intake is indicated as constant, while in the actual experiments, due to the variation in the size of animals, the amount of corn-meal fed varied. In Table I giving the detailed data the relative proportion of milk proteins to corn proteins is the same as shown in the chart. The therms supplied were calculated as *net* available energy.

The results secured are limited to corn-meal (maize) and milk, although they probably are applicable to other cereal grains; but

[2] Hart, E. B., and Steenbock, H., *J. Biol. Chem.*, 1919, xxxviii, 267.

in all probability they would not apply to a grain supplemented with a protein concentrate of plant or animal origin such as oil meal or meat. For example, 600 gm. of corn-meal supplemented with 100 gm. of oil meal may require less milk proteins for efficient nutrition than the level of proteins supplied by corn alone. This inference is made not only because oil meal supplements to some extent the proteins of corn-meal, but because of the higher level of proteins that would be supplied with the corn-meal-oil meal mixture. On the other hand it is known to us[3] that the corn

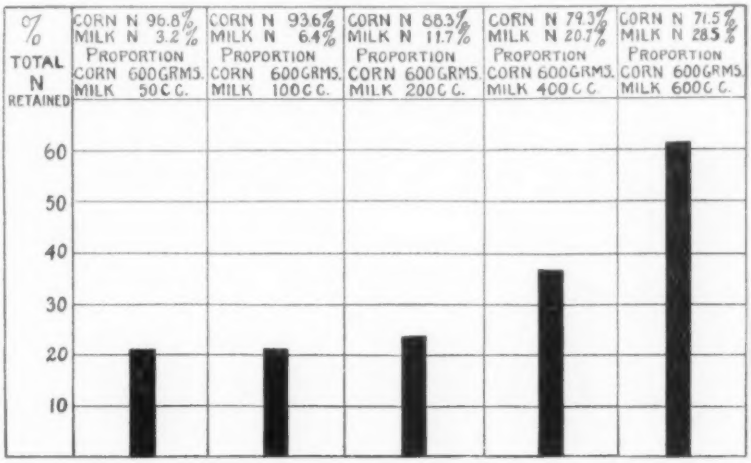

CHART 1. This chart shows the plane at which milk proteins must be used in order to supplement effectively the proteins of corn-meal.

proteins, if they could be fed at a high enough level, would meet the needs of a growing animal.

Accepting Atwater's statement[4] of the daily food intake for growing children of 6 to 15 years of age at 443 gm. of dry matter (2,040 calories) composed of 75 gm. of protein, 43 gm. of fat, and 325 gm. of carbohydrates, the daily fluid intake of milk for an efficient protein mixture could be safely set at 1 pound (equivalent to 454 gm.). This would be liberal from the standpoint of an

[3] Hart E. B., and McCollum, E. V., *J. Biol. Chem.*, 1914, xix, 373. Johns, C. O., Finks, A. J., and Paul, M. S., *J. Biol. Chem.*, 1920, xli, 391.

[4] Atwater, W. O., quoted from Jordan, W. H., Principles of human nutrition, New York, 1917, 182.

TABLE I.

Complete Data on Nitrogen Balance.

Pig No.	Live weight.	Feed daily.	Therms provided daily.	Total N fed daily	Total protein fed daily.	Protein in ration.	Total N fed 24 days.	N in feces 24 days.	N in urine 24 days.	Total N retained.	Absorbed N retained.
	lbs.			*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1	44	600 gm. corn-meal. 50 cc. skim milk.	1.57	8.37	52.8	8.6	200.8	88.4	65.6	23.3	42
2	50	600 gm. corn-meal. 50 cc. skim milk.	1.57	8.37	52.8	8.6	200.8	100.9	47.7	25.9	52
3	47	600 gm. corn-meal. 50 cc. skim milk.	1.57	8.37	52.8	8.6	200.8	105.6	68.0	13.5	29
4	43	600 gm. corn-meal. 50 cc. skim milk.	1.57	8.37	52.8	8.6	200.8	81.8	72.3	23.3	39
Average										21.5	
5	49	700 gm. corn-meal. 117 cc. skim milk.	1.86	10.18	63.6	8.9	244.3	109.7	82.9	21.1	31
6	63	700 gm. corn-meal. 117 cc. skim milk.	1.86	10.18	63.8	8.9	244.3	133.8	80.0	12.5	28
7	51	700 gm. corn-meal. 117 cc. skim milk.	1.86	10.18	63.8	8.9	244.3	106.4	58.9	32.3	57
8	65	800 gm. corn-meal. 133 cc. skim milk.	2.12	11.61	72.6	8.9	278.6	142.2	74.9	22.1	45
Average										22 0	

9	87	800 gm. corn-meal. 266 cc. skim milk.	2.16	12.4	77.5	9.4	297.6	131.9	75.8	30.2	54
10	102	900 gm. corn-meal. 300 cc. skim milk.	2.43	14.0	87.5	9.4	336.2	142.6	107.9	25.6	44
11	104	900 gm. corn-meal. 300 cc. skim milk.	2.43	14.0	87.5	9.4	336.2	174.2	80.3	24.3	50
12	68	800 gm. corn-meal. 266 cc. skim milk.	2.16	12.4	77.5	9.4	297.6	116.7	115.6	21.9	37
Average										25.5	
13	75	800 gm. corn-meal. 533 cc. skim milk.	2.25	13.9	86.8	10.2	333.6	107.9	100.5	37.5	56
14	73	800 gm. corn-meal. 533 cc. skim milk.	2.25	13.9	86.8	10.2	333.6	106.0	99.7	38.4	56
15	80	800 gm. corn-meal. 533 cc. skim milk.	2.25	13.9	86.8	10.2	333.6	118.9	98.4	34.9	57
Average										36.9	
16	90	800 gm. corn-meal. 800 cc. skim milk.	2.33	15.4	96.1	10.9	369.6	57.4	85.4	61.3	72
17	85	800 gm. corn-meal. 800 cc. skim milk.	2.33	15.4	96.1	10.9	369.6	60.4	81.9	61.5	73
18	87	800 gm. corn-meal. 800 cc. skim milk.	2.33	15.4	96.1	10.9	369.6	65.4	80.8	57.7	73
Average										60.2	

efficient protein mixture, not only because of the high plane of protein intake required by the standard, but also because of the possibility that a mixture of proteins from diverse sources would, in themselves, have a fair efficiency. Basing the energy intake for children on the recent studies of Gephart,[5] then the milk consumption should be increased. In fact it would be double that suggested by the Atwater standard since these studies showed a daily intake of 4,000 to 5,000 calories by athletic boys.

From the results presented the inference might be made that normal rates of growth could not be secured until a highly efficient protein mixture was fed. This would appear to be contrary to what has been published in the literature. The apparently successful rearing by Hindhede[6] of his own children on low levels of protein of presumably only fair quality is a point in evidence. Probably the explanation of such results lies in the slower rates of growth of man, coupled with the possibility of increased consumption. Further, recent experiments by Osborne and Mendel,[7] where with rats and a level of 10 per cent of barley protein good growth was secured, make it more difficult to obtain a full and clear comprehension of this problem. In maintenance experiments with man, Sherman and Winters[8] did not obtain a positive nitrogen balance when practically all the protein of the diet came from corn-meal. A negative nitrogen balance was converted into a positive balance, when a certain amount of milk supplemented the corn-meal.

However, from the standpoint of the greatest utilization of ingested protein it would seem that experiments should be directed toward finding the most efficient protein mixtures possible. It would be greater economy to use a mixture of barley protein and milk proteins at levels of effective supplementary relations rather than depend upon excessive intake of a protein mixture of low efficiency, and certainly not successful as the sole source of proteins with all animals, even of the same species.

The positive mathematical facts obtainable with cage experiments and nitrogen retention leave no doubt as to the relative

[5] Gephart, F. C., *Boston Med. and Surg. J.*, 1917, clxxvi, 17.
[6] Hindhede, M., Protein and nutrition; an investigation, London, 1913.
[7] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1920, xli, 275.
[8] Sherman, H. C., and Winters, J. C., *J. Biol. Chem.*, 1918, xxxv, 301.

merits of protein mixtures examined and dispel the constant doubt in growth experiments that increments in weight may not be protein storage alone, but in part may be due to an excessive storage of fat, water, *etc.* From the practical experiences of animal husbandmen in the growth of swine it has been demonstrated again and again that maximum rates of growth cannot be secured with the cereal grains fortified with all other nutritive factors and supplied unconsciously in forage, such as alfalfa pasture. A protein supplement of high efficiency such as tankage or skim milk has always been found necessary for maximum rates of growth. This experience harmonizes with our contention that it is always safer to use highly efficient protein mixtures in nutrition during growth. This by itself justifies the use of milk in the human dietary.

Our views are in agreement with the statement made by Sherman,[9] who has given careful consideration to the problem of the protein requirement of maintenance in man:

> "It is plainly desirable in all cases that grain products be supplemented by milk products, and it is clear that in providing for needs of growing children and of pregnant or nursing mothers the proportion of milk in the diet should be more liberal than it need be when only maintenance is concerned; this both because of the superior amino-acid make-up of the milk proteins and to provide amply for the mineral elements and vitamines as well."

SUMMARY.

The results show that a highly efficient protein mixture is not obtained until the proportion of liquid milk to corn-meal reaches 1:1; in this proportion the milk nitrogen will constitute approximately 30 per cent of the total nitrogen of the ration.

These data relate only to protein efficiency and of course are not to be interpreted as indicating the amount of milk necessary for providing an adequate supply of vitamines or salts to an animal with a growth rate similar to that of swine.

[9] Sherman, H. C., *J. Biol. Chem.*, 1920, xli, 97.

FERMENTATION CHARACTERISTICS OF CERTAIN PENTOSE-DESTROYING BACTERIA.*

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The pentoses, xylose and arabinose, have been termed non-fermentable sugars. In a general sense this is true, as the majority of microorganisms, yeasts and bacteria, cannot utilize the pentoses; however, there are certain forms which possess the ability to break down these sugars. Apparently these pentose-fermenting bacteria are widely distributed and no doubt play an important rôle in the economy of nature.

In previous publications[1,2] attention was called to the occurrence of certain pentose fermenters in soil, manure, sauerkraut, and silage. These organisms were isolated, their general properties described, and the name *Lactobacillus pentoaceticus, n.sp.*, was suggested. This group of the pentose fermenters is characterized chiefly by the rapidity with which it breaks down xylose and arabinose with the production of acetic and lactic acids, and traces of alcohol and carbon dioxide.

The present paper deals with the general fermentation characteristics of the pentose fermenters.

EXPERIMENTAL.

To determine the value of any substance as a source of carbon for an organism it is desirable to measure its growth in a synthetic medium free from any other source of carbon. In the case of the

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[1] Fred, E. B., Peterson, W. H., and Davenport, A., *J. Biol. Chem.*, 1919, xxxix, 347.

[2] Peterson, W. H., and Fred, E. B., *J. Biol. Chem.*, 1920, xli, 181.

pentose fermenters all attempts to grow these organisms in synthetic media failed. After several tests, it was found best to use yeast water medium for all fermentation tests. The yeast water alone contains a very small amount of available carbon compounds, hence a control, consisting of yeast water alone plus the proper organism, was run and in reporting results these controls have been subtracted. The yeast water used in this study of fermentation reactions was prepared as follows:

450 gm. of pressed yeast were steamed 3 to 4 hours with 4,500 cc. of tap water with occasional stirring. This infusion was then allowed to stand undisturbed until a heavy deposit of yeast cells and other suspended matter had settled to the bottom of the vessel. The portion above this deposit was carefully poured off and sterilized in liter flasks. These flasks of the sterile yeast infusion were allowed to stand for 1 to 2 weeks, when the supernatant liquid was syphoned off and used without clearing or filtering.

For a few of the experiments this medium was cleared with egg albumin and a clear yellow liquid resulted. The desired amount of the carbohydrate, 2 per cent unless otherwise stated, was then added; and if calcium carbonate was not to be used in the medium its reaction was adjusted to a hydrogen ion concentration of about pH = 7.0 by titration with phenol red. Accurately measured portions were then pipetted into Erlenmeyer flasks and sterilized at 12 pounds pressure for 30 minutes. The calcium carbonate was sterilized in powdered form and added to the culture medium immediately before inoculation. In many of the cultures, the indicator bromocresol purple was added and the acid produced neutralized by means of sterilized 1 N NaOH; unless otherwise stated, the sugars were sterilized in the yeast water.

The yeast water was found very satisfactory for the growth of the pentose fermenters. All the analyses were made in duplicate and frequently in triplicate, usually with closely agreeing results. Since no variation was noted in the products of fermentation from cultures kept under widely different tensions of oxygen, the organisms were cultivated in deep layers of culture solutions, usually 250 cc. of culture in a 300 cc. Erlenmeyer flask. Continued cultivation in the presence of certain carbohydrates apparently did not produce any change in the power of fermentation. All cultures were incubated at 28°C. for varying periods of time. The carbohydrates used were, with the exception of xylose,[3] Merck C.P. products. The substances studied were:

[3] The xylose was obtained through the courtesy of Dr. C. S. Hudson, formerly Chief of the Carbohydrate Laboratory of the Department of Agriculture.

1. Pentoses: xylose and arabinose.
2. Methylpentose: rhamnose.
3. Hexoses: glucose, galactose, mannose, and fructose.
4. Disaccharides: sucrose, maltose, and lactose.
5. Trisaccharides: raffinose and melezitose.
6. Polysaccharides: starch, inulin, cellulose, and xylan.
7. Glucosides: salicin and esculin.
8. Alcohols: mannitol and glycerol.
9. Organic acids: pyruvic, lactic, succinic, malic, tartaric, and citric.

In all the work a 2 per cent solution of the various carbon compounds was used. Fermentation was indicated by a change in the hydrogen ion concentration and particularly by the amount of volatile and non-volatile acid produced. As a rule these organisms bring about a vigorous fermentation without noticeable gas accumulation in a fermentation tube. This is particularly true of the fermentation of the pentoses, xylose and arabinose. The volatile acid was determined by distilling with steam, and the non-volatile acid by extracting the residue from the steam distillation with ether in a Kutscher-Steudel extraction apparatus for 50 to 75 hours. In a number of cases the barium salts of the volatile acid and the zinc salts of the non-volatile acid were prepared and analyzed.

Additional studies of the chemical changes arising from the decomposition of these carbon compounds are now in progress. A report on this phase of the work will be given at a later date.

A Comparison of the Fermentation of Various Carbohydrates and Related Compounds.

Reaction.—Changes in the hydrogen ion concentration of culture media is frequently used as an index of fermentation. This method of study was employed with many of the common sugars and related compounds fermented in the absence of a neutralizing agent. The pH values were determined 14 days after the different media were inoculated with Cultures 41–11 and 118–8. The control tubes contained yeast water only and were inoculated with the same cultures. They gave a change of not more than pH 0.4. It will be seen from the data in Table I

that all the carbohydrates used were fermented, except raffinose, with a decided change in the hydrogen ion concentration. The fermentation proceeds rapidly, especially in the case of xylose and fructose; and usually within 24 to 48 hours there is a well defined change in pH values. After the 4th or 5th day the true acidity of the xylose, glucose, galactose, and fructose cultures remains practically constant at about pH 3.6 to 4.0. No reversion from an acid reaction to a more alkaline was ever noted.

TABLE I.

Comparison of Acid Production from Various Sugars and Related Compounds.

No.	Carbon compound.	Culture 41-11.			Culture 118-8.		
		At beginning.	At end.	Difference.	At beginning.	At end.	Difference.
		pH	*pH*	*pH*	*pH*	*pH*	*pH*
1	Xylose	5.6	3.6	2.0	5.6	3.5	2.1
2	Glucose *a**	6.0	4.0	2.0	6.0	3.6	2.4
	Glucose *b*	6.8	3.9	2.9	6.8	3.8	3.0
3	Galactose	7.2	4.0	3.2	7.2	4.0	3.2
4	Fructose	6.4	3.9	2.5	6.4	3.9	2.5
5	Sucrose *a*	6.0	3.6	2.4	6.0	3.6	2.4
	Sucrose *b*	7.0	3.8	3.2	7.0	4.6	2.4
6	Lactose *a*	5.8	3.7	2.1	5.8	4.4	1.4
	Lactose *b*	6.8	3.9	2.9	6.8	Lost.	
7	Mannitol	6.6	4.3	2.3	6.6	4.3	2.3
8	Raffinose	7.0	6.6	0.4	7.0	6.4	0.6
9	Yeast water alone	7.0	6.6	0.4	7.0	6.6	0.4

* *a* and *b* denote different experiments.

The rate of acid formation is much slower in the case of sucrose, lactose, and mannitol than in the case of the other carbon compounds and the pH values are higher. The results indicate that the sugars, xylose, glucose, galactose, fructose, sucrose, and lactose, are readily decomposed, giving approximately the same pH limit of growth. The final reaction averages about pH 3.6 to 3.9 which may be termed the acid limit of fermentation for this group of organisms in this media. The mannitol is less easily decomposed and consequently gives a higher pH value, probably due to the slow rate of fermentation.

As has been noted by other investigators[4] it was observed that the greatest changes in hydrogen ion concentration often were accompanied by apparently little visible growth in the tubes. A vigorous fermentation with only a slight visible growth in the culture medium is especially noticeable in the case of xylose.

Undoubtedly changes in pH values furnish an easy means of indicating carbohydrate fermentation, but do not necessarily give a true picture of the utilization of these substances. For instance xylose in concentrations not in excess of 4 per cent is converted almost quantitatively into acetic and lactic acids; while glucose, galactose, and sucrose under the same conditions show only a small utilization of the added sugars.

Gas Production.—The method of studying carbohydrate fermentation by gas production in Smith fermentation tubes was tested. For this purpose 2 per cent yeast water solutions of xylose, glucose, galactose, fructose, sucrose, lactose, raffinose, and mannitol were used. The cultures were kept at 28°C. and examined daily for a period of 3 weeks. During this time no gas collected in the closed arm of any of the fermentation tubes.

Although gas production in a fermentation tube has been used as a common indication of carbohydrate decomposition, for the pentose fermenters this method is almost worthless since only traces of gas are produced. In the majority of cases where the fermentation tubes gave no visible gas production more accurate methods of analysis showed a strong fermentation with gas formation.

Keyes,[5] Clark,[6] and others have pointed out the inaccuracies of the fermentation tube for measuring gas production and the use of the results thus obtained as an index of growth. The decomposition by the pentose fermenters of glucose, galactose, mannose, and other sugars with the formation of CO_2, none of which can be collected in fermentation tube, offers striking examples of the fallacy of this method. In the case of these sugars, no gas is noted in the closed end of the fermentation tube and yet

[4] Ayers, S. H., Rupp, P., and Johnson, W. T., *U. S. Dept. Agric., Bureau of Animal Industry, Bull. 782*, 1919, 21.

[5] Keyes, F. G., *J. Med. Research*, 1909, xxi, 69.

[6] Clark, W. M., *Science*, 1913, xxxviii, 669.

they ferment rapidly with the production of large amounts of CO_2 and organic acids. About 20 to 25 per cent of the glucose and galactose is converted into CO_2.

Comparison of Reaction and Gas Production.

Unfortunately measurements of the change in hydrogen ion concentration also fail to give a true idea of the fermentative value of the carbon compounds. Often the change in pH value may be well defined when only a small amount of the carbohydrate has been consumed. Although far superior to the fermentation tube test as a criterion of fermentation, the change in hydrogen ion concentration is open to certain objections. The change in pH values depends upon many factors, chiefly the amount and strength of the acid or acids formed. An organism may bring about complete utilization of the carbohydrate with the production of large amounts of alcohol, acetone, or other neutral bodies and the relatively weak carbonic acid. On the other hand the carbohydrate may suffer only a slight decomposition with the production of stronger acids, for example, acetic, lactic, and succinic acids, and consequently a well defined change in pH values.

In a study of the fermentation reactions, it is believed that the total acid as well as the kinds of acids should be considered. With this in mind attention was directed to the soluble acid products of fermentation.

In this connection it should be borne in mind that the chemical processes involved in the utilization of carbohydrates by bacteria are by no means simple; and undoubtedly the steps are much more complicated than was formerly supposed. The intermediate and often the end-products are difficult to demonstrate and are subject to changes depending upon the conditions of the fermentation. In this work only the products which accumulated during fermentation were measured. The carbohydrates are considered in the order of their availability and their chemical relation; the most easily fermented are given first.

Fermentation of Xylose, Arabinose, and Rhamnose.

The total amounts of volatile and fixed acids produced at different stages of growth from the pentoses, xylose, arabinose, and rhamnose, in cultures inoculated with 41–11 and 118–8 are tabulated in Table II. Xylose and arabinose are decomposed very rapidly by these organisms with the formation of large amounts

TABLE II.

Fermentation of Pentoses.

	Culture No.	Time.	0.1 N acid in 100 cc. of culture.			
			Total.	Volatile.	Non-volatile.	Ratio of volatile to non-volatile.
		days	*cc.*	*cc.*	*cc.*	
Xylose.	41–11	3	123.7	64.6	59.1	100:91
	41–11	6	194.8	106.4	88.4	100:83
	41–11	8	207.2	115.2	92.0	100:80
	41–11	10	222.7	121.2	101.5	100:84
	41–11	14	226.5	124.0	102.5	100:83
	41–11	90	209.4	118.2	91.2	100:77
	118–8	3	126.8	66.6	60.2	100:90
	118–8	6	196.2	107.6	88.6	100:82
	118–8	8	224.7	130.4	94.3	100:72
	118–8	14	231.5	132.4	99.1	100:75
Arabinose.	41–11	28	234.8	136.6	98.2	100:72
	118–8	28	248.8	138.2	110.6	100:80
Rhamnose.	41–11	30	No fermentation.			
	118–8	30	" "			

of acid. In a previous paper[1] attention was called to the great rapidity and the completeness of the fermentation of xylose by *Lactobacillus pentoaceticus.*

It will be noted from Table II that more than 12 per cent of normal acid was produced in a culture containing 2 per cent of xylose after 3 days incubation and nearly 20 per cent after 6 days. The maximum was found after 10 days of growth, about 22 per cent of normal acid. After 14 days incubation a portion of the culture was removed and the sugar content determined. In

every case it was found that the bacteria had destroyed the pentose sugar.

The interesting fact noted from the data of this table is the relation between the volatile and non-volatile acids. This ratio is approximately 100 parts of volatile acid (cc. of 0.1 N acid) to 82 parts of non-volatile acid. It has been found that the acids formed consist almost exclusively of acetic and lactic acids. Arabinose apparently behaves like xylose, being decomposed rapidly into acetic and lactic acids. On the other hand, the methylpentose rhamnose is not attacked. Here it seems that the presence of the methyl group and the configuration of the hydroxyl groups prevented these organisms from decomposing it.

Fermentation of Glucose, Galactose, Mannose, and Fructose.

The aldo-hexoses, glucose, galactose, and mannose, are decomposed with the production of acetic acid, lactic acid, and ethyl alcohol, while from the ketose, fructose, mannitol is formed instead of ethyl alcohol. The total amount of acid formed, however, is not half that obtained from the pentose sugars, xylose and arabinose. The results of the fermentation of the hexose sugars are given in Table III.

Glucose.—Within 24 hours after inoculation the glucose cultures showed a well marked turbidity, a few gas bubbles, and a well defined acid reaction. The acidity with this hexose amounted to 10 to 14 per cent of normal acid, depending to a certain extent on the age of the culture. The general course of fermentation with glucose is very different from that noted with xylose and arabinose. Acids are formed rapidly in the early stages of fermentation, usually 2 to 3 days after inoculation; and then this period of rapid fermentation is followed by one of slow acid production. Apparently the pentose fermenters can make direct use of only a portion of the glucose. Of the 2 per cent present in the cultures at the beginning, it was found by analyses that more than half of this amount remained at the end of 30 days growth. The same incomplete consumption of sugar also occurs with galactose and mannose but, as already mentioned, to only a slight extent in the fermentation of xylose. Factors other than the accumulation of salts of acids must operate to produce this

noteworthy difference in the behavior of the bacteria toward the hexoses. The sugar consumed correlates with the total acid formed which in the case of glucose is only half as great as that obtained from the fermentation of the pentoses, arabinose and

TABLE III.

Fermentation of Hexoses.

	Culture No.	Time.	0.1 N acid in 100 cc. of culture.			
			Total.	Volatile.	Non-volatile.	Ratio of volatile to non-volatile.
		days	*cc.*	*cc.*	*cc.*	
Glucose.	41–11	15	99.2	16.0	83.2	100:520
	41–11	30	106.5	26.3	80.2	100:305
	41–11	75	149.4	69.4	80.0	100:115
	118–8	15	98.0	15.8	82.2	100:520
	118–8	25	119.9	20.6	99.3	100:482
	118–8	30	100.7	18.6	82.1	100:441
	118–8	75	125.4	54.6	70.8	100:130
Galactose.	118–8	10	63.4	10.7	52.7	100:493
	41–11	30	77.2	16.7	60.5	100:362
	118–8	30	76.6	13.3	63.3	100:476
Mannose.	41–11	14	44.9	16.1	28.8	100:179
	41–11	35	90.7	54.7	36.0	100: 66
	118–8	14	33.9	16.9	17.0	100:101
Fructose.	41–11	14	88.5	52.0	36.5	100: 70
	41–11	21	91.2	53.4	37.8	100: 71
	41–11	28	97.0	60.0	37.0	100: 62
	41–11	42	133.5	72.5	61.0	100: 84
	118–8	14	89.5	51.9	37.6	100: 72
	118–8	42	133.9	74.0	59.9	100: 81
	118–8	4*	68.8	39.9	28.9	100: 72
	118–8	58*	69.7	49.7	20.0	100: 40

* Same culture analyzed after 4 and 58 days.

xylose. Concerning the proportion between the volatile and non-volatile acids, the interesting fact is brought out that in the early stages of fermentation this ratio is about 100 to 500. Apparently this ratio changes in the older cultures, the volatile acid increases, while the non-volatile acid decreases. Although the

evidence is not conclusive, it seems that the non-volatile acid, lactic, is slowly converted into the volatile acid, acetic. The results of fermentation tests with the salts of lactic acid lend support to this assumption. In the pentose sugars no such change in the ratio of acids was noted.

Galactose.—The pentose bacteria ferment galactose in a manner almost identical with that of glucose. From the total acid produced it appears that galactose is somewhat less available than glucose.

Mannose.—This sugar is not fermented in the same way as glucose and galactose. As a matter of fact, mannose occupies a unique position among the hexose sugars whose fermentation has been studied. The decomposition of the mannose is effected much more slowly than is the case with glucose or galactose. The difficult fermentation of mannose may possibly be correlated with the difference in stereoisomeric structure from that possessed by the other aldo-hexoses. As may be seen from Table III, the total acid formed after 14 days is about 3 to 4 per cent of normal acid and the ratio about 100 parts of volatile to somewhat more than 100 parts of non-volatile acid.

Fructose.—Fructose is decomposed in a very different manner from any of the other hexoses. These organisms attack this ketone sugar vigorously, reducing it to mannitol and forming at the same time volatile and non-volatile acids and carbon dioxide. The soluble acid products of fermentation have been found to consist almost entirely of acetic and lactic acids. From sugar determinations it was found that the fructose is completely destroyed within 4 to 6 days after inoculation. It is clear from the results of Table III that the hexose sugars are fundamentally dissimilar in respect to availability; fructose is by far the most easily decomposed; glucose and galactose come next in order; and mannose last. It is significant that, with fructose, mannitol is formed as an intermediate product, and later this intermediate product is in turn decomposed with the formation of soluble acids. For more detailed information on this phase of the subject, see a later report.[7] The ratio of acetic acid to lactic acid in the case of fructose is about 100 to 70.

[7] Peterson, W. H., and Fred., E. B., *J. Biol. Chem.*, 1920, xli, 431.

Fermentation of Sucrose, Maltose, Lactose, Raffinose, and Melezitose.

Next to the pentoses and hexoses in availability as measured by the production of acid come the disaccharides, sucrose, maltose, and lactose. Sterilization of the disaccharides may result in a partial hydrolysis and the production of the corresponding monosaccharides. A 2 per cent solution of sucrose in distilled water heated for 45 minutes at 15 pounds pressure showed a production of 0.012 gm. of reducing sugar per 100 cc. of solution, or 0.006 gm. of reducing sugar per gm. of sucrose. The same quantity of sucrose in yeast water gave 0.008 gm. of reducing sugar per gm. of sucrose. In order to obviate the influence of this reducing sugar, a sucrose solution was sterilized by passing through a Chamberland filter. The bacteria started somewhat more slowly in the latter case but the final acidity developed was essentially the same as in those cultures sterilized by heat. Of these three, sucrose appears the most available, giving an aver-

TABLE IV.

Fermentation of Disaccharides and Trisaccharides.

	Culture No.	Time.	0.1 N acid in 100 cc. of culture.			
			Total.	Volatile.	Non-volatile.	Ratio of volatile to non-volatile.
		days	*cc.*	*cc.*	*cc.*	
Sucrose.	41-11	25	117.8	53.4	64.4	100:121
	41-11	48	190.8	75.4	115.4	100:153
	118-8	25	86.8	29.4	57.4	100:195
	118-8	48	148.8	54.8	94.0	100:172
Maltose.	41-11	16	102.1	29.6	72.5	100:245
	118-8	16	105.6	29.5	76.1	100:258
Lactose.	41-11	25	82.2	33.7	48.5	100:144
	118-8	25	82.2	35.0	47.2	100:135
Raffinose.	41-11	20	No fermentation.			
	118-8	20	"	"		
Melezitose.	41-11	20	"	"		
	118-8	20	"	"		

age of almost 17 per cent of normal acid after 48 days incubation. Maltose is fermented in much the same manner as sucrose, but was perhaps not so available. Too much emphasis should not be placed on the ratio between volatile and non-volatile acids, since the time of incubation was different for the two sugars, sucrose and maltose. Lactose is broken down very slowly by this group of bacteria, giving a much lower acidity than the other disaccharides. The trisaccharides, raffinose and melezitose, show little if any fermentation. Table IV gives the results of these fermentations.

TABLE V.

Fermentation of Alcohols and Glucoside.

	Culture No.	Time.	0.1 N acid in 100 cc. of culture.			
			Total.	Volatile.	Non-volatile.	Ratio of volatile to non-volatile.
		days	*cc.*	*cc.*	*cc.*	
Mannitol.	41-11	15	13.6	7.0	6.6	100:94
	41-11	40	81.6	43.8	37.8	100:86
	41-11	80	114.8	61.0	53.8	100:88
	41-11	140	148.9	75.7	73.2	100:97
	118-8	15	11.2	6.0	5.2	100:87
	118-8	40	79.2	40.2	39.0	100:97
	118-8	80	87.9	46.7	41.2	100:88
	118-8	140	140.0	73.0	67.0	100:92
Glycerol.	41-11	30	9.6	5.0	4.6	100:92
	118-8	30		5.0		
Salicin.	41-11	20	31.7	20.5	11.2	100:55
	118-8	20	25.9	17.1	8.8	100:51
Esculin.	41-11	20	No fermentation.			
	118-8	20	" "			

Fermentation of Mannitol, Glycerol, Salicin, and Esculin.

The polyatomic alcohol, mannitol, furnishes a fair source of carbon for the pentose-fermenting group of organisms. As shown in the results of Table V mannitol is fermented very slowly at first; after 140 days, however, the total acid reaches more than

14 per cent normal. The ratio between the two acids is maintained about the same throughout the entire fermentation period, usually 100 to 86–97. Glycerol was fermented very slowly. It is unsuitable as a source of carbon for these organisms. The glucoside, salicin, is slowly decomposed with the production of about two parts of volatile to one part of non-volatile acid. Esculin was not fermented.

Fermentation of Starch, Inulin, Cellulose, and Xylan.

The cultures containing the polysaccharides, starch, inulin, and cellulose, failed to show any decided increase in acids (Table VI). On the other hand, xylan decomposed very slowly, forming almost equal amounts of volatile and non-volatile acids.

TABLE VI.

Fermentation of Polysaccharides.

	Culture No.	Time.	0.1 N acid in 100 cc. of culture.			
			Total.	Volatile.	Non-volatile.	Ratio of volatile to non-volatile.
		days	*cc.*	*cc.*	*cc.*	
Starch.	41–11	30	No fermentation.			
	118–8	30	"	"		
Inulin.	41–11	30	"	"		
	118–8	30	"	"		
Cellulose.	41–11	30	Trace.	Trace.	Trace.	
	118–8	30	"	"	"	
Xylan.	41–11	60	58.7	27.5	31.2	100:113
	118–8	60		35.3	Lost.	

Fermentation of Salts of Organic Acids.

In order to determine the value of the salts of organic acids as a source of carbon, the fermentation of lactic, pyruvic, succinic, malic, tartaric, and citric acids was studied. In all cases the sodium or calcium salt of the acid was used. The salts of lactic, pyruvic, and malic acids were fermented, but no fermenta-

tion was found in the case of the succinic, tartaric, and citric acid salts. The sodium malate fermentation has been described in connection with the fermentation of fructose and results in the production of lactic acid and carbon dioxide as the chief products together with a small amount of acetic acid. In the case of the lactic acid salt, about 30 per cent of it was converted into acetic acid in 30 days. More complete quantitative data on the fermentation of the organic acids and the relation of these to the fermentation of the sugars will be considered in later papers.

SUMMARY.

The fermentation of various carbohydrates and related compounds was studied by means of the change in reaction and by gas production in fermentation tubes. It was found that the average limit of growth in the yeast water medium was approximately pH 3.6 to 4.0 in the case of xylose, glucose, galactose, fructose, sucrose, and lactose. Mannitol was decomposed more slowly and gave a limit of about pH 4.3. The results of acidity measurements fail to give any idea of the amount of the carbohydrate consumed. The fermentation tube method was tried and found valueless. Apparently the pentose fermenters break down the carbon compound with the production of gas, largely CO_2, which does not accumulate in the closed end of the fermentation tube. In the case of glucose and galactose as much as 27 per cent of this sugar was converted into CO_2 without any accumulation of gas.

In this work special emphasis was placed on the total acid formed from 2 per cent solutions of the various compounds as well as the amount of volatile and non-volatile acid. The pentose sugars, arabinose and xylose, decomposed rapidly forming more than 20 per cent of normal acid, divided into almost equal amounts of acetic and lactic acids. In less than 14 days the xylose and arabinose were completely destroyed. Rhamnose was not attacked by the pentose fermenters.

The aldo-hexoses, glucose and galactose, are decomposed in a similar manner, yielding acetic acid, lactic acid, and ethyl alcohol. However, the total amount of acid produced is small as compared with that from the pentose sugars; approximately 10

to 14 per cent of normal acid is formed. The non-volatile acid is produced in much larger amounts than the volatile; the ratio is about five parts of non-volatile acid to one part of volatile acid. Glucose is perhaps more easily fermented than galactose. The extent of fermentation is about the same with these two sugars; after 15 days approximately one-half of the sugar is consumed.

Mannose ferments more slowly than either glucose or galactose and yields nearly equal quantities of volatile and non-volatile acid.

The ketone sugar, fructose, is rapidly reduced by the pentose bacteria forming mannitol; and at the same time volatile and non-volatile acids and CO_2 are formed. The decomposition of the fructose is very rapid. Within 4 to 6 days after inoculation this sugar has completely disappeared.

The disaccharides, sucrose, maltose, and lactose, are all fermented, but never completely. Of these three substances, sucrose is the most available. Lactose, on the other hand, is broken down very slowly. The products of fermentation are chiefly volatile acid and non-volatile acid, with an excess of the latter. Raffinose and melezitose were not fermented.

The polyhydric alcohol, mannitol, is slowly broken down by the pentose fermenters forming almost equal amounts of the two acids, acetic and lactic. Glycerol and the glucoside, salicin, ferment very slowly, while another glucoside, esculin, fails to show any fermentation.

The polysaccharides, starch, inulin, and cellulose, are not fermented; xylan is decomposed very slowly.

The organic acids, succinic, tartaric, and citric, are not attacked; while pyruvic, lactic, and malic acids are fermented. From lactic acid, acetic acid is produced; and from malic, lactic acid, carbon dioxide, and acetic acid are the chief products.

A PHOTOMETRIC TURBIDIMETER.

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Turbidimeters and nephelometers are instruments designed for practically the same purpose; that is, making quantitative determination on certain substances by estimating the translucency possessed by certain concentrations where a cloudiness exists or can be brought about. Some authorities call their instruments turbidimeters because they measure side scattered light, others call theirs nephelometers because they measure light reflected off at different angles (same phenomenon). The nephelometer is properly so called because it measures the amount of cloudiness in the substances, but the latest models of these instruments measure the amount of light that is transmitted through or is scattered by the cloud which exists in the substance.

In the case of either instrument, the operator arrives at his determination by measuring the amount of light that reaches his eye, whether or not he subtracts this from a total to get the absorbed light.

It occurred to the authors of this paper that in the use of the present instruments the operator is handicapped in two of the essential points. First, he must make a standard for comparison for almost every separate determination; and he must depend on the individual eye for the vanishing point of a light, or the merging of gratings. The present authors found that the latter differed with different eyes. In fact one of us found it differed with the right and left eye. Then with the use of the present instruments, where comparisons to standards are made, a point in the concentration is soon reached where the eye is entirely unable to distinguish between two substances that do not have a comparatively wide difference between them.

In 1900 Whipple and Jackson[1] designed the candle turbidimeter for the purpose of measuring turbidity of water. The candle flame under a certain depth of water would disappear. The turbidity of course was assumed to vary inversely as the depth required to cause the candle to disappear. This principle is used yet to a great extent in field work where electricity is unobtainable and other than arbitrary standards are inconvenient. This mechanism has been improved greatly by Sheppard[2] recently.

Our purpose has been to devise an instrument with which turbidities as great as that made by 1 cc. of a 5 per cent lemon oil extract in 100 cc. of water can be measured photometrically.

The latest models of nephelometers are not efficient to this extent. A history of the development of the nephelometer and a description of one of the later ones were published by Kober and Graves.[3] Dienert,[4] Bloor,[5] and Kober[6] developed the artificial light arrangement on the Duboscq colorimeter. In all these arrangements we must depend on the sensitivity of the eye for results.

Richards and Wells[7] provided aid for the eye in comparing intensities of illumination by means of ground glass wedges on their eyepiece. Kober and Graves[3] provided a sort of Lummer-Brodhun field for this comparison by the use of reflecting mirrors. A similar device was also employed by Marshall and Banks[8] in their instrument. But with all these instruments judgment depends on the ability of the eye to match intensities of light, and concentrations of substances soon go beyond that ability.

It seemed to the authors as though some photometric device such as the oil spot on paper would be more sensitive to slight differences in intensity of illumination.

[1] Whipple, G. C., and Jackson, D. D., *Tech. Quart.*, 1900, xiii, 274.

[2] Sheppard, S. E., *J. Ind. and Eng. Chem.*, 1920, xii. 167.

[3] Kober, P. A., and Graves, S. S., *J. Ind. and Eng. Chem.*, 1915, vii, 843.

[4] Dienert, F., *Compt. rend. Acad.*, 1914, clviii, 1117.

[5] Bloor, W. R., *J. Biol. Chem.*, 1915, xxii, 145.

[6] Kober, P. A., *J. Biol. Chem.*, 1917, xxix, 155.

[7] Richards, T. W., and Wells, R. C., *Am. Chem. J.*, 1904, xxxi, 235.

[8] Marshall, J. T. W., and Banks, H. W., 3rd, *Proc. Am. Phil. Soc.*, 1915, liv, 176.

The apparatus which we have designed and operated, and which we wish to describe here, is fashioned so that a strong light is passed through a definite and comparatively narrow column of the substance in question, and what is not lost falls upon one side of an oil spot to balance with an amount that we permit to come from the opposite side by causing graduated losses on similar sources, under standard conditions. The apparatus consists of a dark box 12½ inches long, 4½ inches wide, and 8 inches high, having a dark compartment at each end for the standard lights, next to these the small dark compartments for the cell of unknown substances on the one side and the standards on the other, a very small compartment in the middle for the paper with its oil spot, and the narrow mirrors which reflect the images of the spot through a small eyepiece directly in front. Fig. 1 is a diagram of the apparatus.

The method of using this instrument will be readily seen. The unknown is put into the cell to the left as soon as prepared according to specified conditions and the eye is placed over the eyepiece in front. (Eyepiece is a wooden drawer knob with widening hole toward the screen and serves to lift lid.) Then switch on lights and turn the wheels, by the portion which extends above slots in lid, until the two intensities of illumination are balanced. Then read the resistance, units on first wheel to the right and tenths on the second. The visible numbers on the exposed portion of each wheel tell the equivalent of standard discs brought opposite the aperture through which the light passes. Each different substance determined has to have a scale of its own worked out previously. Then with the reading obtained, by comparing to the proper scale, percentages can be read.

Fig. 2 shows the construction of the wheels containing the standard discs.

While there might be no limit to the number of wheels with different denominations of discs, our instrument has only two. In our experience we found this to be sufficient for the low percentages that must of necessity be expressed. The percentages can, of course, be run to one or two decimals further either way by using varying amounts of the substance in solution.

In the compartment in which the unknown is placed, on the wall next to the light, a tube of the same size as the apertures for

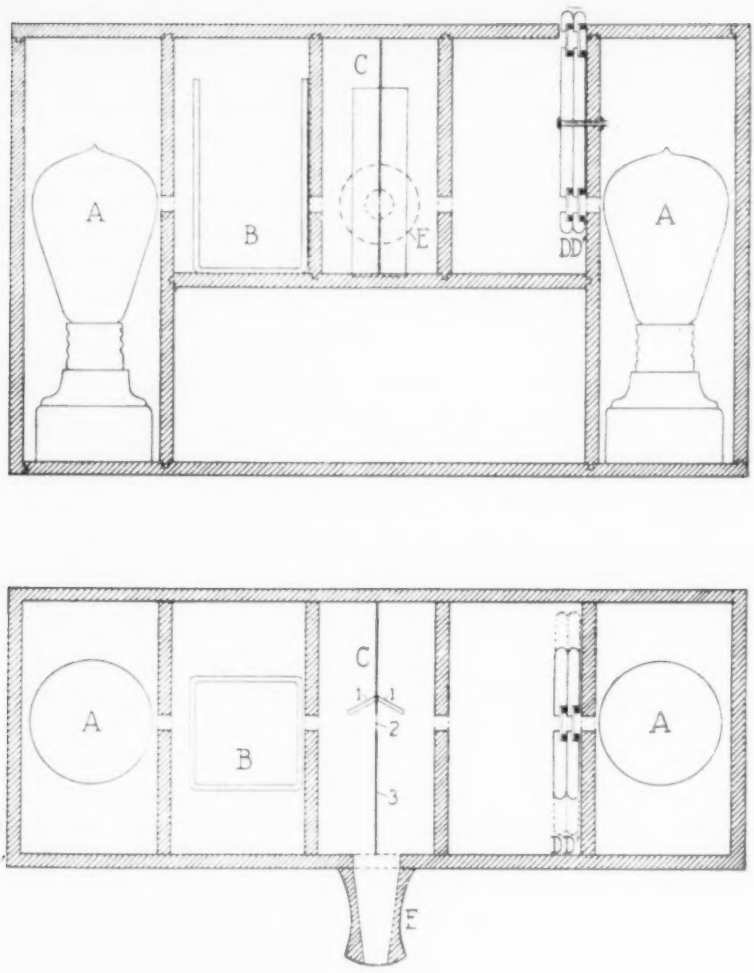

FIG. 1. Upper figure is a diagrammatic view from the side; lower figure view from above.

AA = 50 watt nitrogen lamps joined parallel.
B = Cell to hold substance to be tested.
C = Photometer, (1) reflecting mirrors, (2) oil spot, (3) screen of white paper.
DD′ = Discs holding standards of ground glass.
E = Eyepiece through which the photometer is viewed.

the light, and of a length equal to the thickness of the two wheels, is placed in the light aperture to give the same conditions as to confining the light rays. For special purposes the compartments for the unknown and the disc wheels should each be large enough to contain a standard cell 2 inches thick. However, an inch cell is what we used. When cells smaller than 2 inches are standardized and used they should always be placed in the same position against the wall next the screen compartment. When two cells are used they should be similarly situated in their respective compartments and the zero holes in the disc wheels brought into play.

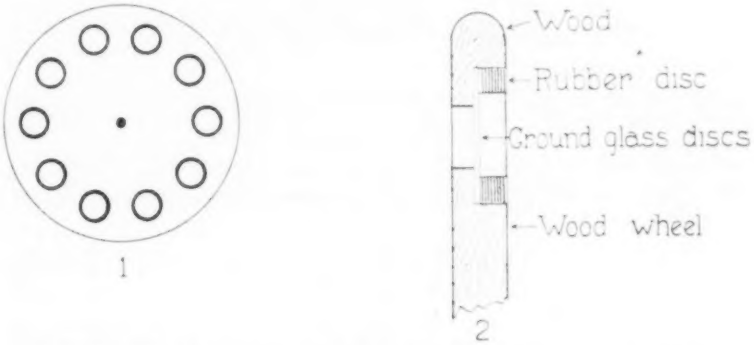

FIG. 2. (1) Lateral view of disc wheel; (2) cross section through discs.

To standardize the disc wheels for a certain substance, we have only to make one standard and place similar amounts in two cells, one for each side of the screen, and adjust the two lights to a zero reading by turning in discs or interchanging lights and turning in discs. Then certain standards which are multiples of each other are made and read and the readings are recorded on a slip which may be filed away to furnish a scale for that same substance in subsequent work. This saves making a new standard for each determination, provided the conditions of preparation are also standardized.

In the use of such an apparatus it is necessary to take cognizance of the work of Richards and Wells,[9,10] who in several articles call

[9] Richards, T. W., and Wells, R. C., *J. Am. Chem. Soc.*, 1905, xxvii, 485. Richards, T. W., *Am. Chem. J.*, 1906, xxxv, 510.

[10] Wells, R. C., *Am. Chem. J.*, 1906, xxxv, 99.

attention to the existence of a progressive change in state of precipitates under different conditions. We can gather encouragement too from Kober[11] in his nephelometric determinations of ammonia, phosphorus, calcium, and the fats and oils; also from the work of Lamb, Carleton, and Meldrum[12] who standardized conditions for chlorine for the nephelometer.

In view of some facts recorded by Wells[10] we could not claim to be able to operate this instrument with all sorts of precipitates, but we hope that with several of the substances which have already been standardized for turbidimetry we can multiply the accuracy and save the time required for making new standards for each determination. We believe also that a great many more additions can be made to the list of substances determinable by the turbidimeter.

TABLE I.

SO_4 in 100cc. H_2O.	Readings by observers.			Average reading.
	A.	B.	C.	
mg.				
10	20	20		20
12.5	24	25	25	24.66
13	25	25		25
13.5	26	26		26
14	27	27		27
15	29	30	31	30
50	48		49	48.5

In order to test the efficiency of the instrument as compared to others we tried two different substances—chlorides as precipitated with $AgNO_3$ and $BaSO_4$ as obtained by 0.1 N H_2SO_4 and $BaCl_2$ in excess. We found that Whipple and Jackson,[1] Dienert,[4] and Muer[13] had operated the nephelometer and turbidimeter on sulfates in water. But Muer was able to determine within only 5 per cent of the true amount when the concentration reached 50 mg. of SO_3 to 100 cc. of water.

[11] Kober, P. A., *J. Ind. and Eng. Chem.*, 1918, x, 556.

[12] Lamb, A. B., Carleton, P. W., and Meldrum, W. B., *J. Am. Chem. Soc.*, 1920, xlii, 251.

[13] Muer, H. F., *J. Ind. and Eng. Chem.*, 1911, iii, 553.

We worked out several different tables in the adjustment of our standards that proved the efficiency of the apparatus, but Table I represents results obtained by three different observers.

Observers A and B were the same persons each time. Observer C was a different person each time. If these were unknown substances supposed to contain sulfate and each case was assumed to be a 1 per cent solution of the unknown solid the average reading would be 1, 1.23, 1.5, and 2.43 per cent. The last, the one most in error, would be off 0.075 per cent. About 1 hour was occupied in the reading of each of these, each observer taking at least three sittings before giving his ultimate readings. At the end of each ½ hour for the following 1½ hours Observer A made observation on each preparation and found that they deteriorated at the rate of 0.1 per cent per ½ hour. We then made the 25 mg. standard with 25 cc. of glycerol and 75 cc. of water and made observations every ½ hour for 3 hours. We found that it then read 35 instead of the 24.66 reading obtained in the water suspension, that it remained 35 throughout six successive readings, and then fell at the rate of about 0.1 per cent each hour. We found in all readings that we could stir the precipitate just previous to taking the reading without changing the reading at all, and the above described deterioration occurred regardless of the stirring, and was doubtless due to agglomeration.

We operated the instrument then on AgCl preparations according to the method of Lamb, Carleton, and Meldrum[12] and found that it was only after working considerably with standards that we could compare light intensities with the yellow color on one side and white on the other. Then taking several readings made by three independent observers, each taking at least three sittings, we obtained the data given in Table II.

We note here that the concentration 2.65 mg. of chlorine to 100 cc. of solution, which was pronounced too concentrated by Lamb, Carleton, and Meldrum, was read the same by Observers A, B, and C and that one-fifth more than this concentration, which is about three times the concentration which they pronounced best, has not so great a deviation from the average as they had with the smaller concentration.

After taking the above reading we performed a few experiments placing fractional portions of the colored substance on the stand-

ard side of the instrument and making up the balance by turning of discs. We got very good results with a much higher concentration than was used in Tables I and II.

We believe that with an instrument in which the difference between a standard and an unknown can be made up with known additions to the weaker one, as is the case with the Marshall-Banks instrument and the one we are here describing, that the above scheme will not only save the making of standard substances to compare to the unknown but it will serve the purpose better. It seems to avoid errors that might creep in on account of the unknown's having more of some interfering substance than does the standard. We are now working out more definite data

TABLE II.

Chlorine in 100cc. water and alcohol.	Readings by observers.			Average reading.
	A.	B.	C.	
mg.				
2.65	25	25	25	25
3.18	30	30	31	30.33
6.36	58			58
10.0	90			90

in this connection and also working on the problem of neutralizing the color or other interfering conditions by bringing colored substances or colored glasses into the path of the light, and reducing the light on the side of the standard[14] with a rheostat resistance in order to obtain more accurate reading in the presence of colors. We hope also that ultimately a colorimeter may be made of this same apparatus.

SUMMARY.

The photometric oil spot is applied to the turbidimeter to balance intensities of illumination, in measuring turbidities. A device is made in which thin columns of substance can be used and a system of standards is arranged.

[14] Suggested by Professor W. F. Sudro of this laboratory.

THE CHEMICAL ISOLATION OF VITAMINES.

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The existence of at least three vitamines, namely the antiscorbutic, the so called fat-soluble, and the antineuritic, is known at the present time. This paper deals principally with the antineuritic vitamine.

The important stage in the chemical investigation of this substance began with the classical research of Eijkman in 1897, who was able to show that rice polishings contain the antineuritic vitamine and that this substance is dialyzable and not precipitated from its solution by alcohol. Eijkman's work stimulated Funk to attempt the chemical isolation of the substance. In a series of researches he established the following facts. First of all he found that the curative substance is of a simple nature, as yeast can be hydrolyzed for 24 hours with 25 per cent sulfuric acid without causing the destruction of its vitamine. The active substance is precipitated by phosphotungstic acid and is mainly found in the silver nitrate-baryta fraction, when subjected to silver precipitation. This fraction contains three classes of biological products; namely, the histidine, pyrimidine, and nicotinic acid groups. On working up these fractions from yeast Funk isolated three substances which on analysis yielded the following formulas: $C_{24}H_{19}O_9N_5$, $C_{23}H_{23}O_9N_5$, and $C_6H_5O_2N$ (nicotinic acid). By using the same or similar methods, Suzuki, Shimamura, and Odake, Vedder and Williams, and Edie, Evans, Moore, Simpson, and Webster were also able to isolate more or less active vitamine fractions from active raw material. Similar fractions were obtained by Funk from milk, brain, lime juice, rice polishings, and cod liver oil.

The fact that the antineuritic vitamine is precipitated from its solution by reagents, which as a rule precipitate basic substances, and the observation that the active, purified fraction contained nitrogen led Funk to the adoption of the term vitamine, a terminology which future work may perhaps prove to be incorrect from the standpoint of chemical nomenclature.

The work of the chemical isolation of vitamine up to 1913 had thus lead to a considerable increase in our knowledge of the chemical character of the antineuritic vitamine, but satisfactory methods for the preparation of the substance in a pure form and in large amounts were still lacking. In 1913 the United States Public Health Service extended its investigations of the etiology of pellagra on account of the menacing increase in this disease in the southern states. One of us (Voegtlin) began a study of the nutritional conditions in these districts, followed early in 1914 by a chemical study of the possible etiological factors of this disease. That the disease might be due to the deficiency of certain factors of the vitamine type as suggested by Funk had been early recognized by Voegtlin, who undertook extensive animal experiments in this connection. With the same purpose in view, work was also begun in this laboratory on new methods for the preparation of highly active vitamine preparation in relatively large quantities for the treatment of pellagra. As the first result of this work, Seidell succeeded in obtaining a very active preparation by treating filtered autolyzed yeast with fullers' earth. This active preparation can be obtained easily on a large scale and was used for the treatment of pellagra patients by Voegtlin, Neill, and Hunter.

In the experimental work, part of which the authors of the present paper are about to describe and which covered a period of several years, brewer's yeast was also made use of as a raw material for the antineuritic vitamine. Nearby breweries furnished an ample supply of bottom yeast, which was pressed into a cake by means of a filter press before additional fermentation had taken place.

The yeast was then placed in a hot room (40°C.), in alcohol barrels as containers, and allowed to autolyze. 200 cc. of chloroform were used for every 100 pounds of yeast. Autolysis was usually complete in about 36 hours, at which time the thin liquid

was run through the filter press yielding a clear dark liquid with a specific gravity of about 1.01. This liquid was used as the source of material in the early work, even though it was finally found to be very unsatisfactory on account of its chemical complexity.

Extraction with Olive Oil.

When autolyzed yeast filtrate is treated with concentrated hydrochloric acid, a heavy, flocculent precipitate is obtained. It was found in connection with the yeast filtrates used in this laboratory that 40 cc. of concentrated acid were necessary for complete precipitation of 1 liter of filtrate. This material is easily filtered off and amounts to about 4.35 per cent of glue-like material, giving the common protein reactions. In all the work which is described in the following pages, the yeast filtrate was treated in this manner. It was found that this filtrate could be kept for a period of at least 2 years without losing its physiological activity, that it can be used for oral administration in the treatment of polyneuritis by neutralizing the acid with sodium hydroxide, and that it represents a suitable raw material for adsorption isolation experiments when the hydrogen ion concentration is adjusted by means of sodium borate.

In view of the fact that in nature the antineuritic vitamine is closely associated with lipoids, the idea suggested itself that this vitamine might be soluble in fats.

Autolyzed yeast filtrate was shaken with olive oil on a shaking machine until an emulsion was formed, with 1 cc. of olive oil for each 4 cc. of yeast filtrate. The emulsion was allowed to stand until two distinct layers were formed and by means of a separatory funnel the oil layer was separated. The oil was filtered to remove a small amount of sediment and then taken up in eight or ten volumes of ether. 0.1 per cent hydrochloric acid was used for the extraction of the ether solution. The acid extract was slightly pigmented and was concentrated in vacuum. A heavy precipitate was obtained with phosphotungstic acid, giving a deep blue color after the addition of sodium carbonate. An insoluble precipitate is formed with picric acid. The biuret

test is negative. The extract promptly relieves the polyneuritic symptoms of pigeons.

The same results are obtained by the use of oleic acid instead of olive oil.

Dried Yeast.

After several unsuccessful attempts to remove impurities from the products obtained by extraction, the following method was used with a considerable degree of success. Dried brewer's yeast was selected as the source of material. The yeast as it was obtained from the brewery was pressed until it crumbled easily between the fingers. It was broken into small particles and dried with a current of air at ordinary temperature, which required 36 to 48 hours. This dried product retains its activity for many months if stored in a dry place.

Dried yeast prepared in this way was ground in a mill repeatedly until a very fine powder was obtained. This was placed in a balloon flask of suitable size with reflux condenser, and extracted with 95 per cent methyl alcohol in the proportion of 2 cc. of alcohol for each gm. of yeast. 1 cc. of concentrated hydrochloric acid was added for each liter of alcohol used in the extraction. The contents of the flask were heated to boiling on the water bath for 3 hours and the soluble part was filtered off by means of suction. The residue was washed once with 1 cc. of hot methyl alcohol per gm. of yeast. The yeast was then extracted a second time using the same proportions of solvent. The extracts were combined and the alcohol was removed in vacuum at 35°C. The wax-like residue was repeatedly extracted with ether and 0.1 per cent hydrochloric acid, the volume being kept as small as possible. The final acid extract should never exceed 2 cc. for each gm. of yeast. The acid aqueous extract is always tested on polyneuritic birds for its activity and then it is purified as described below.

Hot aqueous silver acetate is added to the extract until precipitation is complete by testing a small portion in a test-tube. This purine precipitate, which also contains AgCl, is carefully washed with distilled water. Only a small amount of the active material passes over into this purine fraction.

A large excess of silver acetate was then added to the filtrate from the purine precipitate, followed by saturated barium hydroxide solution until the mixture was distinctly alkaline to litmus. The precipitate was filtered and carefully washed with cold distilled water. Considerable precipitate is produced in this manner, and additional precipitate may be obtained by further addition of baryta.

The silver-baryta precipitate containing the vitamine and some other extraneous material was suspended in water and made distinctly acid with sulfuric acid. The precipitate was then decomposed with hydrogen sulfide with occasional stirring in order to break up any lumps. A slightly pigmented filtrate was obtained after removing the silver sulfide. The excess hydrogen sulfide was removed in vacuum and the filtrate was then treated with a slight excess of lead acetate to remove the sulfuric acid. The lead was removed by hydrogen sulfide and the filtrate concentrated in vacuum at 35°C., with a small amount of ethyl alcohol to aid the distillation. Up to this stage practically none of the activity is lost. This concentrated solution of vitamine and impurities was then treated with mercuric sulfate prepared according to the directions of Kossel and Patten. A pale yellow precipitate was formed upon the addition of the mercuric sulfate, which was filtered off and washed with a small amount of ice water. This precipitate represents the histidine fraction and does not contain active material. The filtrate containing an excess of mercuric sulfate was treated with absolute ethyl alcohol until precipitation was complete. This precipitate was pale yellow and contained the bulk of the active material. The degree of separation in this last procedure is influenced by two factors, (1) the concentration of excess mercuric sulfate present and (2) the final concentration of ethyl alcohol. If these adjustments are right, none of the active material remains in the alcoholic filtrate. The alcohol-insoluble precipitate was suspended in water and the mercury was removed by means of hydrogen sulfide. The filtrate from the mercuric sulfide was freed of hydrogen sulfide in vacuum. The sulfuric acid was removed by means of lead acetate, and the excess lead by hydrogen sulfide.

By eliminating the first lead treatment the process might be shortened, but it was found that this would lead to considerable pigment passing over into this fraction.

This solution was concentrated in vacuum at a low temperature. This fraction gives a purple solution when tested with ninhydrin. Phosphotungstic acid gives a heavy precipitate, slightly soluble in an excess of the reagent, with only a slight blue color upon the addition of saturated sodium carbonate. With diazotized sulfanilic acid and sodium carbonate a reddish brown color was obtained. The biuret test was negative. The solution does not yield a precipitate with picric acid. The solution was highly active when tested on polyneuritic birds. If the solution is concentrated in vacuum, over soda lime, a definitely crystalline product is obtained which shows activity as long as the crystals are surrounded with the mother liquor. The material was soluble in methyl alcohol and yielded a soluble hydrochloride. As soon as the crystals are washed with absolute ethyl alcohol and dried, the physiological activity is lost and the crystal form changes from spindles to prisms. When the prisms are redissolved in a relatively large volume of water and again allowed to crystallize, the spindle-shaped crystals are reformed. It seems probable that there are at least two substances in the final solution both of a distinctly basic character. Work is being continued on this subject, the material being tested as a protective product. It is realized that the activity may be in the non-crystallizable mother liquor as well as in the crystals.

One of the impurities is a histamine-like substance, as shown by the positive Pauly reaction. Voegtlin and Myers have recently shown that the active fractions possess physiological activity when introduced intravenously into dogs. There is a fall of blood pressure and a stimulation of pancreatic and bile secretion following the injection of this material. It is possible that this stimulating action on pancreatic secretion and the fall in blood pressure are actually due to the presence of traces of histamine or a histamine-like substance in these fractions, as according to Abel and Kubota histamine is very widely distributed in animal tissues and probably also in vegetable cells.

SUMMARY.

Autolyzed yeast filtrate on account of its complexity represents an unsatisfactory material for the chemical isolation of the antineuritic vitamine. Mastic, Lloyd's reagent, and ferric phos-

phate, in experiments which, because of their negative outcome, it has not seemed desirable to detail, have been found unsatisfactory adsorbing reagents because they lack specificity. These reagents remove also inactive basic material which cannot be separated by our present methods from the active material. Olive oil and oleic acid remove the antineuritic substance from autolyzed yeast filtrate, thus showing that it is fat-soluble as well as water-soluble in the form of a crude extract. Stachhydrine, trigonelline, and allied betaines show no antineuritic activity. Histidine and its esters are likewise inactive.

The active material is readily extracted from dried yeast by means of acid methyl alcohol. It can be purified by use of the Funk silver method and the mercuric sulfate procedure, yielding an apparently crystalline active substance. This substance becomes inactive upon drying and it is believed that impurities still remain which can be removed with additional modifications of the method described in this paper. The present method eliminates purines, histidine, proteins, and albumoses, leaving a liquid that can be crystallized and probably contains histamine or histamine-like substances. The physiological action of the active fractions resembles that of extracts obtained from the mucosa of the small intestine, when the intestinal and yeast extracts are purified in the same manner.

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OSMOTIC PRESSURE OF SERUM AND ERYTHROCYTES IN VARIOUS VERTEBRATE TYPES AS DETERMINED BY THE CRYOSCOPIC METHOD.

WITH A NOTE ON THE EFFECT OF FREEZING ON THE ARTERIAL BLOOD OF THE RAT.

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It is generally assumed that the osmotic pressure of animal cells is approximately equal to that of the body fluids which bathe them. Thus according to Mathews (1), "Animal cells presumably have an osmotic pressure approximately equal to that of the circulating liquids like the blood."

It would appear that the blood itself is an ideal tissue in which to study relations in osmotic tension between the cells and the surrounding fluid medium, the plasma, or in the case of defibrinated blood the serum. The results of a series of determinations of the freezing points of whole blood (defibrinated), blood serum, and corpuscles of various vertebrate types are recorded in Table I. In this series of papers we have accepted the assumption that the difference, Δ, between the freezing points of a solution and that of water is a measure of the osmotic pressure of the solution.

The blood samples were obtained in various ways, but in every instance the sample corresponded to the arterial type, before the readings were taken. In the case of the sheep the samples were drawn from the external jugular vein of the normal animal. The samples of dog blood were taken from the femoral vein while the animal was under light ether anesthesia. Ox blood and pig blood were obtained from the abattoir at the time the animals were bleeding freely from the carotid artery and jugular vein. Samples were obtained from rabbits, birds, and turtles by bleeding from the carotid, the animal being first stunned by a blow on the head. The

samples of human blood were taken from the median basilic vein. All samples were either aspirated directly into a thick walled, clean, dry, pressure filter, receiving flask containing clean, dry, glass beads, or else caught in the same by allowing the blood to trickle through a paraffined glass funnel. The samples were

TABLE I.

Type.	Δ serum.	Δ defibrinated blood.	Δ corpuscles.	Difference between Δ serum and Δ corpuscles.
	°C.	°C.	°C.	°C.
Sheep	0.621	0.599	0.582	0.039
	0.575		0.538	0.037
Rabbit	0.615	0.585	0.570	0.045
	0.665	0.625	0.607	0.058
Dog	0.647		0.581	0.066
	0.630	0.585	0.605	0.025
Cat	0.68		0.605	0.075
	0.69		0.645	0.045
Man	0.585	0.555	0.520	0.065
	0.655		0.617	0.038
	0.615	0.586	0.563	0.052
Pig	0.600	0.585	0.575	0.025
	0.605	0.585	0.575	0.030
	0.601	0.585	0.582	0.019
Rat	0.619		0.582	0.037
Kitten	0.636		0.612	0.024
Ox	0.598	0.568	0.547	0.051
Fowl	0.650	0.640	0.630	0.020
	0.652		0.618	0.034
Pigeon	0.633		0.590	0.043
	0.690		0.621	0.069
Turtle	0.522		0.480	0.042

shaken in the open flask for about 5 minutes. In those instances in which the Δ of whole blood was determined, the sample for this purpose was taken at this point. The defibrinated blood was at once centrifuged, the serum and corpuscles were pipetted off into clean, dry tubes, and readings taken immediately. When blood samples were obtained at the abattoir a delay of about 2 hours occurred between the time of sampling and defibrinating, and the centrifuging. In as far as the Δ of a physiological fluid is a reliable index of the osmotic pressure of the same, it follows from the results of this series of experiments that the osmotic pressure of the contents of the red cells is in all instances lower than that of the corresponding serum, while that of the whole blood falls between these two values. Stewart (2) found that

TABLE II.

Experiment No.	Δ serum.	Δ corpuscles.
	°C.	°C.
1	0.607	0.577
2	0.539	0.519
3	0.591	0.573
4	0.586	0.570

the Δ for the whole blood was 0.628°, that for corpuscles 0.597°C. Moore and Roaf (3), working with the blood of the pig, found that the Δ for the serum was always slightly higher than that of the erythrocytes. Their results are given in Table II.

They also found that when pig corpuscles were allowed to come into equilibrium with various salines, and afterwards separated from the same by centrifuging, the Δ for the cells was always in excess of that of the saline. The result of one such experiment by these authors follows.

	°C.
Δ original saline solution	0.983
Δ after admixture and centrifuging	0.847
Δ corpuscles after admixture and centrifuging	0.788
Difference	0.059

The results which are reported in Table I would therefore corroborate the findings of Moore and Roaf (3), as regards the

variation in osmotic tension between the red cells and serum of the pig. This difference in osmotic pressure between the erythrocytes and the serum, which in the case of the pig blood was found to be comparatively small, varies considerably in the different types studied.

The maximum difference found between the Δ for serum and corpuscles is 0.075°C. in the case of cat, the minimum being 0.019°C. in pig. The average difference in the twenty-two experiments quoted is 0.043°C. Assuming that differences in Δ represent actual differences in osmotic pressure, the above average difference in Δ would present a difference of approximately 400 mm. of mercury between the osmotic pressure without, and within, the red cell. The depression of the freezing point of the centrifuged corpuscles was found in no instance to be equal to or greater than that of the corresponding serum. This being the case it is therefore established, providing always that the cryoscopic method is a reliable index of osmotic pressure, that there is a definite and appreciable difference in the osmotic pressure of the red cells and the serum of the types studied. One would seem justified also in concluding from the data furnished by these experiments that this variation in osmotic pressure between red cells and serum, which are apparently in osmotic equilibrium, is general throughout the vertebrate class.

It was found that laking of the corpuscles by repeated freezing and thawing did not have any appreciable effect on the Δ unless, however, one started with venous blood, in which case the depression was less due to loss of carbon dioxide but after equilibrium was established the reading remained constant. A typical example of this is quoted.

	°C.
Sheep blood, red cells (venous) first freezing.....	0.562
" " " " " laked by alternate freezing and thawing, and agitated for 15 minutes with stirrer.	0.536

The effect of the carbon dioxide content on the freezing point was also tested in another manner. Blood drawn from the jugular vein of a sheep was divided into two portions after defibrination. Oxygen gas was bubbled through the one sample for 15 minutes, and through the other carbon dioxide gas was passed for a like period of time. The serum and corpuscles were then separated

by centrifuging and the depression of the freezing point of the respective serum and corpuscles was determined. The results are as follows:

		°C.
Sample made arterial by oxygen gas	Cells	0.59
	Serum	0.64
" " venous by carbon dioxide gas	Cells	0.65
	Serum	0.70

Kovács (4) has previously shown that the addition of carbon dioxide to rabbit blood raised the Δ from 0.60 to 0.72°C.

The effect of freezing at all temperatures from −6 to −40°C. of oxygenated corpuscles was found to be negligible.

It is possible that the differences in osmotic pressure between serum and corpuscles which are indicated by these cryoscopic studies do not exist under the conditions holding in the blood stream of the normal animal.

The ease with which crystalline oxyhemoglobin of the rat blood can be obtained was constantly demonstrated in the freezing of the erythrocytes of this animal. While there was slight tendency for the oxyhemoglobin of other bloods to crystallize on freezing, it was found that in the case of the rat cells practically the whole mass of oxyhemoglobin crystallized out with a single freezing, and that, instead of showing the characteristic laky appearance on thawing, the rat cells were pinky red in color as viewed in the glass container. Microscopic examination revealed the fact that almost complete crystallization of the oxyhemoglobin had occurred, many crystals being contained in the red cell envelopes. Rat erythrocytes which had been frozen could be mixed with several volumes of distilled water with only a trace of hemolysis being manifested, and the bulk of oxyhemoglobin crystals could be recovered again by centrifuging or allowing sedimentation to take place. Rat cells made venous in type by bubbling carbon dioxide gas through them previous to freezing were laked in the usual manner.

SUMMARY.

The results of the determination of the depression of the freezing points of whole blood, blood serum, and corpuscles in various vertebrate types are recorded. The Δ for corpuscles is always found to be lower than for the corresponding whole blood or serum.

The average difference in osmotic tension between the red cells and the serum in twenty-two determinations as calculated from the data obtained by the cryoscopic method is approximately 400 mm. of mercury.

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EFFECT OF DILUTION ON THE OSMOTIC PRESSURE AND THE ELECTRICAL CONDUCTIVITY OF WHOLE BLOOD, BLOOD SERUM, AND CORPUSCLES.

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It has been shown by the author in Paper I that the osmotic pressure of the red cells is appreciably lower than that of the serum in all bloods studied. In the present paper there are reported a number of experiments in which the effect of dilution on the osmotic tension, and on the electrical conductivity of whole blood (defibrinated), blood serum, and corpuscles has been studied.

The effect on the Δ of blood serum, whole blood, and blood corpuscles of dilution with an equal volume of distilled water is shown in Table I. It is found that, whereas in the case of blood serum the value for the Δ is decreased approximately 50 per cent as a result of twofold dilution, in the case of the corpuscles twofold dilution causes a decrease of from 57 to 60 per cent in the Δ. The effect of twofold dilution of whole blood was determined in a few cases only. The decrease in the depression of the freezing point of whole blood diluted with an equal volume of distilled water is greater than in that of the serum similarly treated, but less than that of the corpuscles diluted twofold.

It might be expected that in the case of a weak solution of electrolytes twofold dilution would result in the reduction of the osmotic tension of a unit volume to slightly over one-half the tension of the original solution. The increase in the degree of the dissociation as a result of dilution would cause a relative increase in the number of ions and thus the tension of the diluted solution would be more than half that of the original solution. The results indicate that blood serum behaves somewhat in this manner and probably whole blood and corpuscles do also, although

the primary survey of the readings would not so indicate. As blood corpuscles contain approximately 30 to 40 per cent total solids, it is evident that when one dilutes 100 volumes of red cells with 100 volumes of distilled water, one obtains not a two-

TABLE I.

Type.	Δserum.	Δserum diluted twofold.	Δdefibrinated blood.	Δdefibrinated blood diluted twofold.	Δcorpuscles.	Δcorpuscles diluted twofold.	x
	°C.	°C.	°C.	°C.	°C.	°C.	
Man	0.615	0.337	0.586	0.265	0.563	0.244	76.5
Sheep	0.608	0.309			0.520	0.223	76.0
Rabbit	0.615	0.320	0.585	0.277	0.570	0.240	72.7
	0.625	0.318			0.588	0.254	77.8
Dog	0.647	0.327			0.581	0.254	77.6
	0.630	0.330			0.585	0.255	77.2
Cat	0.685	0.345			0.605	0.255	72.8
Pig	0.600	0.315			0.575	0.242	72.6
	0.601	0.315	0.585	0.265	0.582	0.245	72.1
Rat	0.619	0.310			0.582	0.232	69.1
Kitten	0.636	0.322			0.612	0.259	73.3
Ox	0.598	0.306	0.568	0.276	0.547	0.220	67.2
Fowl	0.652	0.330	0.618	0.295	0.618	0.242	64.3
3 pigeons	0.690	0.343			0.621	0.262	73.0
	0.633	0.343			0.590	0.248	72.5
Turtle	0.522	0.255	0.512	0.231	0.480	0.192	66.6

fold dilution of the fluid fraction of the corpuscles but a much greater one. Thus if we assume that 25 per cent of the corpuscle is, as it were, framework and 75 per cent liquid, then on diluting 100 cc. of red cells with 100 cc. of distilled water one actually dilutes the 75 cc. of liquid in the cells to 175 cc., so that the dilu-

tion is 3 to 7. If one does not allow for increase in the degree of dissociation of the electrolytes with twofold dilution or shrinking in actual volume occupied by the non-liquid portion of the red cell then the following formula should apply $\Delta_1 = \left(\frac{100 + x}{x}\right)\Delta_2$

where x = actual liquid in the red cell,
Δ_1 = depression of freezing point for red cells, and
Δ_2 = depression of freezing point for red cells diluted with an equal volume of distilled water.

When this arbitrary formula is applied to the results recorded for Δ's of red cells before and after dilution for purposes of comparison, one finds that the value for x varies from 66.6 per cent in the case of turtle cells to 77.8 per cent in the case of rabbit cells, while in the case of most of the non-nucleated types of red cells the value for x approximates closely to 75 per cent. Irrespective of in what state one may consider the protoplasm of the red cell to be (fluid, semirigid, or rigid), there is a certain fraction of its bulk which may be considered as framework, and it is the total bulk minus this factor which is in osmotic equilibrium with the surrounding serum. This latter fraction is approximately 75 per cent of the volume of the red cell. The framework (the term used advisedly) would include the cell membranes, the stroma, and, what would be most important, the disperse phase of the colloidal complex within the cell. The liquid phase or the dispersion medium might be considered as holding in solution the molecular and ionic dispersoids contained within the cell membrane with the exception of any such portion of the latter class which may be present in the adsorbed state.

The electrical conductivity of whole blood, blood serum, and erythrocytes of various vertebrate types both undiluted and diluted to various degrees was determined. The Kohlrausch method was employed. Readings were taken at 37°C. and the specific conductivities calculated. The figures given in Table II are for specific conductivities multiplied by 10^5.

As has been shown by Moore and Roaf (1) and others, the specific conductivity for serum is several times that for corpuscles. This is due very largely to the mechanical effect of the want of homogeneity of the fluid in the electric field, as shown by Moore and Roaf (1). The fact that the specific conductivity for cor-

TABLE II.

Specific Conductivities at 37° C.

Type.	Undiluted.	Degree of dilution.				
		2-fold.	4-fold.	8-fold.	16-fold.	32-fold.
Human serum	1,582	857	450	242	129	65
" defibrinated blood	693	441	294	184	103	62
" corpuscles	47	145	154	119	71	43
Cat serum	1,714	964	505	270	140	71
" defibrinated blood	873	475	330	194	109	59
" corpuscles	94	137	187	133	71	43
Dog serum	1,543	869	475	242	126	64
" defibrinated blood	475	338	254	160	92	52
" corpuscles	45	124	167	124	76	47
Rabbit serum	1,542	845	457	242	125	65
" defibrinated blood	616	470	300	220	114	62
" corpuscles	82	160	208	152	96	54
Sheep serum	1,582	869	471	242	126	64
" defibrinated blood	893	546	346	197	106	58
" corpuscles	133	206	206	131	87	47
Pig serum	1,582	857	463	240	125	63
" defibrinated blood	725	514	331	187	110	59
" corpuscles	75	192	229	158	80	48
Fowl serum	1,648	884	494	257	131	67
" defibrinated blood	694	605	350	212	110	60
" corpuscles	69	140	111	111	65	43
Pigeon serum	1,581	833	460	252	131	66
" defibrinated blood	585		363	199	112	62
" corpuscles	60	147	173	147	90	46
Turtle serum	1,323	685	369	198	102	53
" defibrinated blood	822	514	301	176	93	48
" corpuscles	81	171	182	143	78	44

puscles diluted several-fold with distilled water is always lower than the specific conductivity for serum or defibrinated whole blood at the same dilution would suggest that the ionic concentration within the corpuscle is less than that in the serum. It may be possible, however, as Moore and Roaf (1) have pointed out, that due to the adsorption of crystalloid by the colloid of

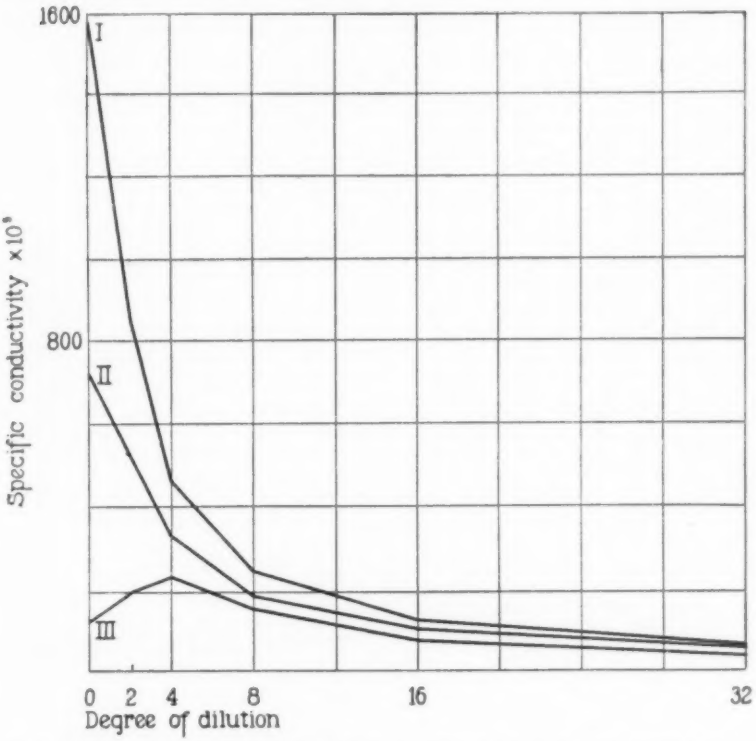

FIG. 1. Pig blood at 37°C. Curve I = serum; II = defibrinated blood; III = red cells.

the hemolyzed red cells the movement of the ions in the electric field is so inhibited that the conductivity is much lower than it would be if the same electrolytes were held in solution in an equal volume of distilled water. What is probably of greater significance, however, is the viscosity factor. This unfortunately could not be determined. The viscosity of the diluted corpuscles would

no doubt be in the earlier stages of dilution appreciably higher than that of the sera of equivalent dilution.

The percentage of inorganic matter, exclusive of iron, in the red cell was found by Schmidt (2) to be lower than that in the serum, 1,000 gm. of corpuscles yielding 7.128 gm. of ash, while 1,000 gm. of serum gave 8.574 gm. of ash. Taking into account

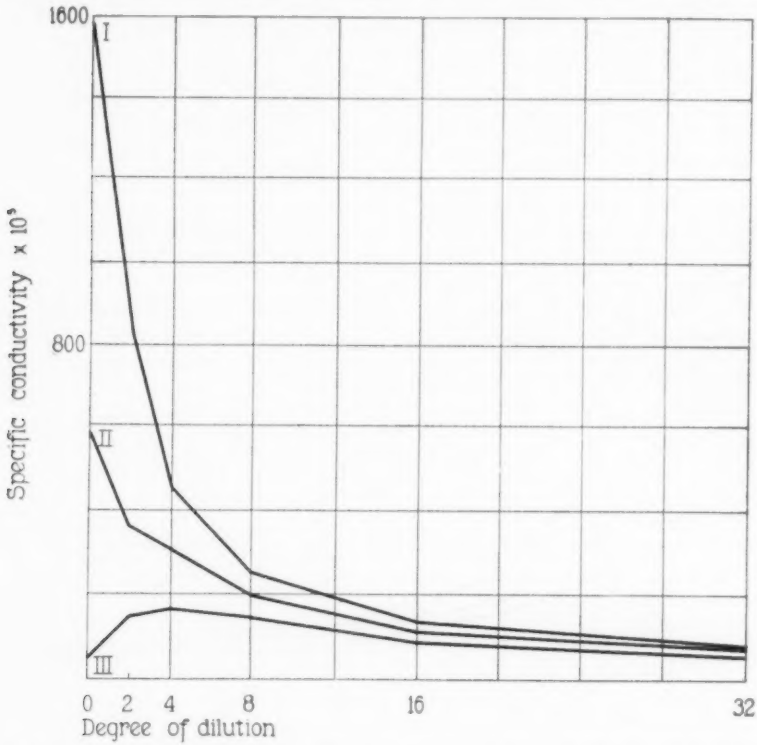

FIG. 2. Pigeon blood at 37°C. Curve I = serum; II = defibrinated blood; III = red cells.

the specific gravity, 1.088 in the case of red cells, 1.029 in the case of serum, these figures would be corrected on the basis of percentage volume to 7.74 gm. of ash per 1,000 cc. of corpuscles and 8.82 gm. of ash per 1,000 cc. of serum—still a marked difference.

Results of quantitative analyses such as these would favor the view that the total electrolyte content of the erythrocytes per unit volume is less than that of the serum.

If we take into account the point urged earlier in this paper that a certain definite portion of the red cell protoplasm may be considered as framework and, as such, actually reduces the fluid volume to approximately 75 per cent of the total bulk of the red cell, then it would follow that the concentration of electrolyte in this fluid must be equal to, if not greater than, that in

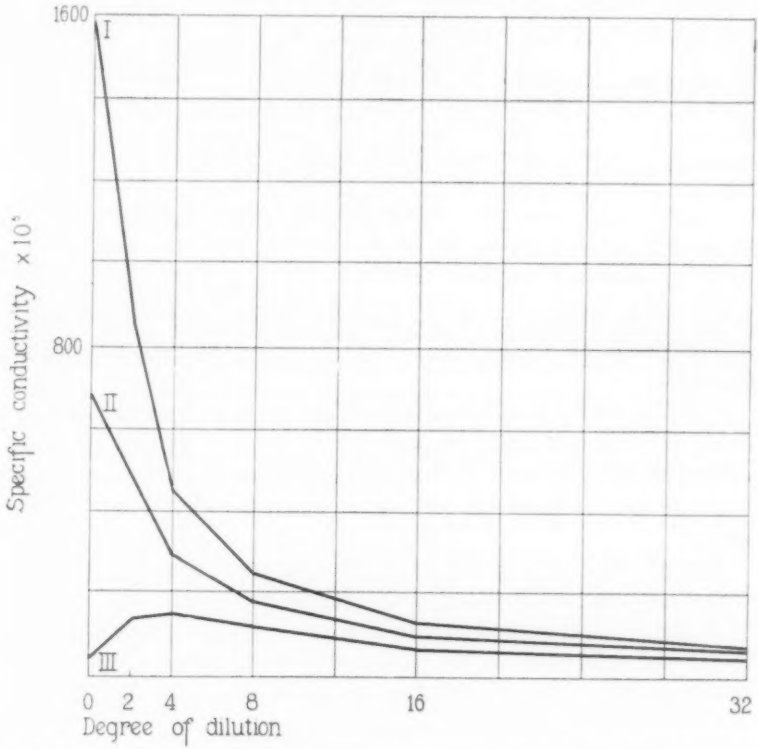

FIG. 3. Human blood at 37°C. Curve I = serum; II = defibrinated blood; III = red cells.

the serum, unless an appreciable part is in a state of adsorption at the membrane surface or with colloidal aggregates.

Preliminary freezing, and thus laking, of the corpuscles causes an increase in their conductivity, as Moore and Roaf (1) have shown. Preliminary freezing prior to twofold dilution was shown to have a like effect. This is no doubt due to the fact that two-

fold dilution in most instances does not cause complete hemolysis, whereas a combination of freezing and twofold dilution does. Figs. 1, 2, and 3 indicate that the conductivity of the red cells rises until hemolysis is complete, after which it falls in much the same manner as the serum. Table III indicates the effect of freezing on the conductivity of the corpuscles.

TABLE III.

Type.	Conductivity × 10^5 of corpuscles alone.		Conductivity × 10^5 of corpuscles diluted twofold.	
	Unfrozen.	Frozen.	Unfrozen.	Frozen.
Rabbit	82	95	160	228
Pig	75	117	192	260

SUMMARY.

The effect of dilution on the osmotic pressure and the electrical conductivity of whole blood, blood serum, and corpuscles is recorded.

Twofold dilution of serum causes approximately a 50 per cent decrease in the osmotic pressure of the same. Twofold dilution of erythrocytes of both nucleated and non-nucleated types causes a decrease of 57 to 60 per cent of the original osmotic pressure to occur.

Tables and charts are shown indicating the changes in conductivity of serum, whole blood, and erythrocytes on dilution.

A greater degree of concentration of electrolyte in serum than in the red cells seems to be indicated although the concentration of electrolyte in the liquid phase of the cell may be equal to, if not greater than, that in the serum.

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OSMOTIC PRESSURE OF TISSUE AS DETERMINED BY THE CRYOSCOPIC METHOD.

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It has been shown by Sabbatani (1) that the osmotic pressure of tissue can be determined by the simple expedient of packing the tissue about the bulb of the Beckmann thermometer contained in the usual form of freezing tube. The tissue, after it has supercooled to a certain degree, suddenly freezes, and the highest point to which the mercury rises in the capillary tube of the thermometer is read off, the Δ thus being determined as in the case of liquids. This method has been applied by Moore (2) in a study of the effects of fatigue on the osmotic pressure of the muscle tissue of the frog.

The constant deviation of the osmotic pressure of the erythrocytes from that of the serum in various vertebrate types observed by the author, and dealt with in detail in Paper I, is suggestive that such differences in osmotic tension might be met with elsewhere, such as between blood and tissue fluid or lymph, or between tissue fluid and the cytoplasm of the cellular elements of the various tissues. The results of the experiments which are reported in this paper suggest that there are actual differences in the osmotic tension of the various tissues, and that of the blood and lymph, and also that there may be an actual difference in osmotic tension between one tissue and another.

The Δ for lymph is as a rule practically the same as that for serum, but slightly higher than that for defibrinated blood. One may therefore assume that the osmotic pressure of the tissue fluid which bathes the cells is approximately the same as that of the blood.

The material studied by the cryoscopic method in this investigation consisted of various tissues and the whole blood of the cat and dog. The blood was aspirated from a large vein while the animal was under ether anesthesia, and defibrinated by shaking with glass beads in an open flask. The tissues were taken when possible while the animal was anesthetized, but failing this they were removed as speedily as possible immediately after death. The tissues were passed at once through a meat grinder, and from 20 to 30 gm. of the minced tissue transferred to the freezing tube, and the Δ was determined immediately. The results of four experiments are given in Table I.

TABLE I.

Tissue.	Experiment 1. Dog. Δ	Experiment 2. Dog. Δ	Experiment 3. Cat. Δ	Experiment 4. Dog. Δ
	°C.	°C.	°C.	°C.
Whole blood	0.61	0.64	0.63	0.82
Lymph	0.63			
Heart	0.77	0.81	0.86	
Lung	0.77	0.74		0.86
Spleen	0.77	0.75		0.87
Liver				0.91
Muscle (thigh, abductors)	0.84	0.85		0.94
Brain		0.76		

It will be noted that in all instances where the Δ for a tissue has been determined it is invariably higher than that for defibrinated blood, the difference being very marked in each case.

In Experiments 1, 2, and 3, the animals were under ether anesthesia, while in Experiment 4 the animal was placed under ether anesthesia, then 20 per cent ethyl alcohol in 0.9 per cent sodium chloride was given intravenously, and the ether discontinued. The samples were taken after a period of 5 hours of alcohol anesthesia. The high Δ recorded for whole blood was due in this instance to the alcohol held in solution in the blood.

It is freely admitted that the Δ for tissue determined in the manner outlined above may not be an absolute index of the osmotic pressure of the same in the intact animal. The difference between the Δ of tissue and that of blood is such, however, that

a higher osmotic pressure within the tissue cell than without would seem probable. This it may be noted is the reverse condition to that found to exist between the red blood cells and the serum.

It is possible also that a certain portion of the molecular and ionic constituents of the tissue which effect the depression of the freezing point of the same are inactive osmotically within the living tissue, being held in restraint in the form of adsorption layers at the interfaces as suggested by Macallum (3).

Another point which has to be considered is the actual volume of fluid in the tissue cell. It is this which must be in osmotic equilibrium with the tissue fluid without.

It has been shown in Paper II that twofold dilution of red cells causes a decrease of from 57 to 60 per cent in the osmotic pressure while in serum diluted twofold the Δ is lowered 50 per cent. It has been found that tissue reacts similarly to the erythrocytes on dilution as the results reported in Table II indicate.

TABLE II.

Experiment 1. Cat. Δ		Experiment 2. Dog. Δ	
	°C.		°C.
Muscle	0.98	Heart	0.74
" diluted twofold	0.39	" diluted twofold	0.31

This is a decrease in the Δ as a result of twofold dilution with distilled water, of 60 per cent in Experiment 1 and of 58 per cent in Experiment 2. The application of the arbitrary formula suggested in Paper II $\Delta_1 = \Delta_2 \frac{(100+x)}{x}$ gives in the first instance a value of 66 per cent for actual fluid in the tissue, and 72 per cent in the second instance. If the osmotic tension of the fluid within the cell is approximately the same as the tissue fluid or lymph without, it follows that the quantity of electrolytes held in the tissue, but removed from the sphere of osmotic activity, must be considerable. It has been pointed out by Moore and Roaf (4) that osmotic equilibrium between two media does not necessarily imply that the osmotic tension of each is identical. It is quite possible therefore that the osmotic tension within the tissue cell is greater than without, and at the same time a condition of os-

motic equilibrium may exist between the cytoplasm of the cellular elements of the tissue and the tissue fluid. The Δ for tissue is higher than that for blood, due chiefly to the fact that there is more non-electrolyte in the former than in the latter. This is illustrated by the following experiment.

A dog was placed under ether anesthesia, and a sample of venous blood taken; this was defibrinated at once. The thorax was then opened, and the heart excised while still beating. The heart freed of blood, as far as possible, was at once frozen solid by placing in a −20°C. atmosphere out of doors. The frozen heart was then allowed to thaw. It was finally passed through a meat chopper, and the minced tissue was weighed, and mixed thoroughly with four times its weight of distilled water. The mixture was divided into two portions A and B. The Δ of an aliquot of Sample A was then determined, while the remainder of this specimen was allowed to stand in a cool place for 2 hours. It was then centrifuged, the supernatant fluid pipetted off, and its electrical conductivity determined at 37°C. by the Kohlrausch method. Specimen B was autoclaved at 117°C. for a period of 30 minutes. It was then centrifuged and the Δ and electrical conductivity of the clear supernatant fluid were determined.

The specimen of defibrinated blood was diluted with four times its weight of distilled water, and then autoclaved for 30 minutes as in the case of Specimen B of heart tissue. The Δ and electrical conductivity of the supernatant fluid obtained after centrifuging were also taken. The results are shown in Table III.

TABLE III.

	Sample A.	Sample B.	Blood.
Δ heart muscle and blood, °C.	0.158	0.159	0.142
Δ " " " " specific conductivity × 10^5	343	363	425

It will be noticed that autoclaving did not produce an appreciable difference in the Δ while the specific conductivity of the clear fluid obtained on centrifuging was definitely increased. This latter effect has probably a double cause due to the removal of the bulk of the colloidal material as a result of coagulation of the protein, and the more thorough extraction of the tissue. This increase in the specific conductivity while definite is, however, not relatively very great. Such wide differences as regards electrical conductivity of extracts between tissue and blood as

are noted in the last experiment quoted were not always manifested. The specific conductivity of the blood extract was, however, in all cases higher than that of tissue extract, while the Δ for the former was less than that of the latter, as the results of a typical experiment indicate (Table IV).

TABLE IV.

Dog under Ether.

Extract plus four times weight of distilled water.	Δ	Specific conductivity $\times 10^6$.
	°C.	
Blood	0.148	456
Pancreas	0.188	395
Muscle (thigh, abductors)	0.177	386
Spleen	0.185	411
Kidney	0.188	425
Heart	0.183	427

The fact that the Δ for tissue is higher than that for blood and also that the Δ for tissue mixed with a definite amount of distilled water is greater than that for blood similarly treated, indicates a higher concentration of total solutes in the tissue than in the blood. On the other hand the lower specific conductivity of tissue extracts, made as described above, compared with blood extracts so made, probably indicates that the free electrolyte in the tissue is not in excess of that of the blood, or that it may be even less in amount. A further experiment, the results of which indicate that the total solute concentration in the tissue is higher than that of the blood, is quoted.

The heart of a cat was excised, weighed, minced, and mixed with four volumes of distilled water. The mixture was autoclaved for 30 minutes at 117°C. It was then filtered, and an aliquot volume of the filtrate reduced on the water bath to an aliquot weight of the original tissue. The Δ for this was then taken; it was 0.75°C., a reading much higher than is ever given by normal cat blood.

It may be urged that in all the experiments quoted sufficient autolysis might occur during the period which elapses between the time of sampling and the actual recording of the depression of the freezing point to account for the difference between the Δ of

tissue and that of blood. While there can be no doubt that a slight increase in the Δ would be caused by autolysis, yet it is the opinion of the author that the differences observed in the Δ for tissues and that for blood are such as to indicate that a definite difference in actual solute concentration does exist between the cytoplasm of the tissue cells and the blood or lymph. The presence of amino-acids in the tissue in much greater concentration than in the blood (5) would account in part at least for this difference in concentration with regard to the total solutes.

SUMMARY.

The Δ for tissue is higher than that for blood or lymph.

The specific conductivity of tissue extracts is lower than that for the blood extracts similarly prepared.

The results recorded point to a higher osmotic tension within the tissue cell than in the blood or lymph.

The concentration of non-electrolyte is greater in the tissue cell than in the blood or lymph.

The concentration of electrolyte is probably greater in the blood than in the tissue.

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MAINTENANCE OF OSMOTIC PRESSURE WITHIN THE NUCLEUS.

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PLATES 1 TO 3.

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It is generally understood that there is a state of osmotic equilibrium existing between the blood plasma and the tissue fluid from which it is separated by the endothelial wall of the capillary, and between the tissue fluid and the cytoplasm of the tissue cells which are separated by the limiting layer of the individual cells. It is also apparent that a condition of osmotic equilibrium must exist between the cytoplasm of the tissue cells and their respective nuclei. It has long been known that the amount of any one element or salt may vary in different tissues. We may take for example the difference in concentration of sodium and potassium in the serum and in the red cell. Microchemical investigations by Macallum (1) have shown that there is a definite distribution of certain ions in each tissue, and the type of distribution of one particular ion in one tissue is not necessarily the same as that in another. Moore, Roaf and Webster (2) have attributed the specific differences, both quantitative and qualitative, in the electrolyte content of different tissues to special affinities between colloids of various types and electrolytes of various types.

It has been found by the author in a microchemical study of various tissues, following the methods evolved by Macallum (1), that the nucleus is free from potassium, chlorine, phosphate, and carbonate ions, thus confirming the observations of Macallum. Results such as these suggest one of two things: either the nucleus is electrolyte-free, or the technique followed in the course of such microchemical investigations is not sufficiently perfect to demonstrate electrolyte in all instances where it actually occurs.

The tissue to be studied was removed from the animal while under ether anesthesia or else cut away as quickly as possible after the animal had been stunned by a blow on the head. A small block of the tissue was then transferred to the chilled stage of a freezing microtome which was inclosed in an asbestos-lined box fitted with a plate glass cover. The process of freezing was almost instantaneous. Distilled water was used to make an ice embedding about the block of tissue. Ten micron sections were cut, a chilled knife being used. The sections were taken up on camel's hair brushes, previously moistened with distilled water and subsequently frozen, and dropped while still frozen into the reagent which was contained in a porcelain basin within the freezing chamber. The air within the freezing chamber was kept well chilled by means of an additional carbon dioxide spray. This latter was also used for chilling the sectioning knife.

When this technique is followed one obtains very clear-cut localizations of potassium, chlorine, phosphate, and carbonate, such as have been described by Macallum (1) and the nuclei throughout appear to be free of electrolyte.

The evidence such as is furnished by microchemical investigation points to the absence of the common inorganic electrolytes from the nucleus. It may be suggested that failure to obtain a reaction in the nucleus for ions which have been localized elsewhere in the tissue may be due to non-penetration of the reagent into the nucleus on account of impermeability of the nuclear membrane. The answer to such a criticism would be that in the method of sectioning outlined above one is certain to cut through nuclei on several occasions and in such cases the permeability of the nuclear membrane to the precipitating reagent, such as cobalt sodium hexanitrite, or silver nitrate, could not be considered as a factor modifying any nuclear reaction which might otherwise occur.

The writer is therefore of the opinion that the results of microchemical investigations carried out as outlined above are adequate proof of the existence in the nucleus of only the slightest traces of inorganic electrolyte. This would seem to be a condition of marked significance, for, if the nuclear fluid is in osmotic equilibrium with the cytoplasmic fluid and therefore has an osmotic tension approximate at least with that of the latter, this tension

must be maintained for the most part by non-electrolyte or organic electrolyte. It would follow from this that, since the concentration of electrolyte in the nucleus is minimal, concentration of non-electrolyte must here be maximal.

It has been shown in Paper III of this series that the concentration of free electrolyte in tissue is probably slightly less than in blood serum, but it is nevertheless true that the osmotic tension of the tissue as a whole is largely determined by the free electrolyte contained in the same. The nucleus, however, if electrolyte-free, must contain non-electrolyte in concentration equivalent to that of an approximately $\frac{1}{3}$ molar solution.

A number of photomicrographs and drawings of tissue sections which have been treated according to the technique previously outlined, with a view to localizing potassium, chloride, phosphate, and carbonate ions, are shown in Figs. 1 to 14. The nuclei of the tissue cells are in practically every instance conspicuous, due to the almost total absence of any reaction in them.

It has been shown by Macallum and Collip (3) that there is in ganglionic nerve cells a substance (probably an hydroxy-benzene derivative) which will reduce silver nitrate in neutral solution. This substance is confined to the cytoplasm of the nerve cells of vertebrate types studied, while in the nerve cells of the leech it appears to extend out into the axon for a short distance (Fig. 1). The entire absence of any reaction with nitrate of silver in neutral solution within the nucleus is the point to be emphasized. This is shown quite distinctly in Fig. 2. The tissue in this instance is a terminal sympathetic ganglion adjacent to the cortical substance of the suprarenal gland. Camera lucida drawings (Figs. 3, 4, and 5) of ganglionic nerve cells which had been treated with silver nitrate in neutral solution also illustrate the absence of any nuclear reaction. Fig. 6 illustrates the distribution of chlorides in ganglionic nerve cell and fiber. It has been shown (4) that chlorides are present in the parietal cells of the gastric tubules in considerable quantity during the stage of active secretion, while during the resting stage they are present in these cells in traces only. In either phase, however, the nucleus is chloride-free. It was also demonstrated that phosphates and carbonates are at all times present in the parietal cells, irrespective of whether they are in the active or resting stage. Figs. 7 and 8 illustrate the distribution of chlorides in the active gastric tubule of the dog.

The luminal half of the cells lining the tubules of the proventriculus of the hen was found to show changes somewhat similar to those noted in the parietal cells of the mammalian stomach in regard to chloride content during the resting and active stages, but here the localization is much more clearly defined, both as regards chlorides, and phosphates and carbonates. Figs. 9, 10, and 11 illustrate the peculiar type of localization. The nucleus is free from any reaction.

At no time were the nuclei of any tissues studied found to be impregnated with ions of potassium, chlorine, phosphate, or carbonate so as to give an appreciable reaction.

It has been demonstrated by Macallum (5) that the cytoplasm as well as the nucleus of nerve cells is potassium-free. Fig. 12 illustrates the distribution of potassium in the Gasserian ganglion of the dog. Brown (6) has shown that the nuclei of the cells of the renal tubule are potassium-free while Defries (7) found no potassium in the nuclei of the salivary or pancreatic gland cells. Macallum (1) has found the nucleus free from all inorganic salt in tissues studied by him.

It is, of course, impossible by microchemical methods to prove absolutely the absence of free inorganic electrolyte from the nucleus. The fact, however, that the ions of chlorine, phosphate, and carbonate are uniformly absent or present in traces only would point to the absence of balancing cations such as those of sodium, potassium, calcium, and magnesium. The absence of potassium from the nucleus is all the absolute data which is yet available as regards its cation content.

The data which have been presented would seem sufficient justification for the theory that the nucleus is very low in total inorganic electrolyte content if not entirely electrolyte-free.

Assuming that such is the case, what evidence is there at hand which will indicate the means by which the osmotic tension of the nucleus is maintained?

The non-electrolytes or organic electrolytes which might possibly occur in greater concentration within the nucleus than in the cytoplasm, tissue fluid, or blood plasma are dextrose, urea, ammonium salts, organic acids (chief among which would be free amino-acids), and other organic substances of low molecular weight.

A certain amount of useful data would be furnished by making quantitative analyses for these various substances in the various tissues. Tissues in which the nuclei are large and make up an appreciable part of the total tissue bulk could be compared with tissues in which cytoplasm is far in excess of the nuclear elements.

It has been found, using the Greenwald method (8), that the total non-protein nitrogen of avian blood is from three to five times as concentrated in the red cells as in the plasma, whereas in mammalian blood the differences noted are of an absolutely different type. Similarly using the Van Slyke and Meyer alcohol method (9) for estimating amino-acid nitrogen, it was found that the nitrogen gas evolved in 3½ minutes at 22°C. from the alcohol-free extract of plasma and erythrocytes of avian blood, calculated as amino-acid nitrogen, gave a value from three to five times as great for the erythrocytes as the plasma, whereas in mammalian blood the amino nitrogen in the corpuscles was approximately the same as in the plasma. The results of these analyses are shown in Table I.

Constantino (10), using the formal method of Sörensen, found that mammalian serum and corpuscles during fasting are constant in their amino-acid nitrogen. He found 4.4 mg. of amino-acid nitrogen per 100 gm. of dog serum and 3.2 mg. per 100 gm. of ox corpuscles. Van Slyke and Meyer (9), using the alcohol precipitation method, found 3 to 5 mg. of amino-acid nitrogen per 100 cc. of blood of dogs which had been fasting 20 to 24 hours. Constantino (10) reports finding 10 mg. of amino-acid nitrogen in 100 gm. of blood obtained from dogs during full digestion. Bock (11), using his own method, found an average of 7.47 mg. of amino-acid nitrogen per 100 cc. of dog blood and 8.43 mg. per 100 cc. of pig blood. Constantino (10) found 3 mg. of amino-acid nitrogen in 100 gm. of turkey blood and 34 mg. in 100 gm. of corpuscles. Bock (12) reports the amino-acid content of bird blood as roughly three times as high as that of mammals. Thus he found the whole blood of the chicken to contain 23.79 mg. of amino nitrogen per 100 cc. of blood divided as follows: 7.79 mg. in the plasma and 16 mg. in the cells. Bock (11) found 20.99 mg. of amino nitrogen as the average for 100 cc. of chicken blood, 21.32 mg. per 100 cc. of duck blood, 20 mg. per 100 cc. of turkey blood, and 18.6 mg. per 100 cc. of goose blood. Wilson and Adolph (13) found the amino nitrogen of whole blood of fish to vary from 17 to 34 mg. while that in the plasma varied from 7 to 18 mg.

In my own analyses the blood was oxalated as it was drawn. It was then centrifuged and the plasma and corpuscles were at once pipetted off into separate graduates. The results reported in

Table I may be a little low as, according to Bock (12), the alcohol precipitation method of Van Slyke and Meyer (9) is not absolutely reliable due to adsorption of some of the amino-acids by the precipitated protein. The urea was not removed in the cases here reported nor was any calculation made to determine the amount of this constituent.

TABLE I.

	Amino-acid N per 100 cc.		Total non-protein N per 100 cc.	
	mg.	*mg.*	*mg.*	*mg.*
Hen cells	37	45	126	
" serum	9	7	35	
Duck cells	34		128	
" serum	6.5		24	
Pigeon cells	36		125	
" serum	11.5		35	
Turkey cells	36	33*	133	
" serum	6	10*	26	
Sheep cells	5		35	
" serum	5		23	
Dog cells			45	
" serum			31	
Goose cells	35*	35*	135*	120*
" serum	11	12	42	29

*In full digestion.

When the actual bulk of the nucleus is taken into consideration there is sufficient non-electrolyte or organic electrolyte (assuming the excess of total non-protein nitrogen in the nucleated red cells over that in the corresponding plasma to be largely confined to the nucleus) within the nucleus to maintain an osmotic tension equal to that of the surrounding cytoplasm where the osmotic tension is, in large measure, due to inorganic electrolyte.

That there is a definite nuclear membrane, the permeability of which must enter into any consideration of exchanges between

nucleus and cytoplasm, is well established. The author has been successful in dissecting nuclei free from nerve cells and photographing the same while under the ultramicroscope. Fig. 13 shows two such nuclei, the cytoplasm of which has been torn away, in the same field with a damaged nerve cell, the nucleus of which is still intact. The source of these nerve cells was a ventral ganglion of the medicinal leech. The absolute homogeneity of the nucleus as viewed under the ultramicroscope stands in sharp comparison with the heterogeneity of the cytoplasm. Fig. 14 shows two normal nerve cells as photographed under the ultramicroscope.

One finds in the case of tissue other than blood that the concentration of total non-protein nitrogen is far in excess of that in the blood and that the same is true of amino-acid. Van Slyke and Meyer (14) have found that the concentration of amino-acid in the tissue is normally from five to ten times that in the blood. They hold that the process by which amino-acids are taken up and held by tissue cannot be wholly osmotic and suggest either the possibility of mechanical adsorption of the amino-acid by the tissue or the formation of loose molecular compounds between the amino-acids and the tissue proteins.

While there is no absolute justification for applying the conclusions reached as regards the maintenance of the osmotic tension of the nucleus of avian blood cells to nuclei of tissue generally, yet it would appear to the writer, in the light of microchemical studies and of quantitative analyses of tissue, that in the nuclei of any tissue there is a higher concentration of total non-protein nitrogen and also of amino-acid than in the cytoplasm, the tissue fluid, or the blood. If the cell membrane is freely permeable to amino-acid, it is difficult to understand why there should be such a marked difference in the content of amino-acid in the blood and tissue. If, however, a large percentage of the tissue amino-acid could be shown to be confined to the nuclei, then one would not only be able to explain the difference in concentration in the amount of this substance in the blood and tissue but one would add an additional function, as well as significance, to the free amino-acid of the tissue; namely, that of acting in a purely physical manner, helping to maintain the osmotic tension of the nucleus.

If the intact nucleus is free of inorganic salts, it would be of great interest to know whether electrolyte gets in among the chromosomes during the stage of mitosis. The writer hopes to have some information on this point shortly. It may be stated here, however, that chlorides, phosphates, and carbonates are absent from the entire cell in the case of the sprouting root tips of the onion where mitotic figures are very much in evidence. The writer is not, however, overconfident of these results, as the usual method of sectioning could not be applied in this instance and the possibility of non-penetration of the silver nitrate reagent into the cell was not disposed of in this experiment. The results are nevertheless suggestive.

SUMMARY.

The freedom of the nucleus from inorganic electrolyte such as chloride, phosphate, and carbonate of potassium is demonstrated.

That there is not only a condition of osmotic equilibrium existing between the blood plasma and the tissue fluid, and the tissue fluid and the cytoplasm of the tissue cells, but also an osmotic equilibrium between cytoplasm and the nucleus in each nucleated cell is pointed out.

As the osmotic tension of the nucleus cannot be maintained by inorganic electrolyte, it is argued that non-electrolyte or organic electrolyte must account for the nuclear osmotic tension.

Evidence is given to show that the concentration of total non-protein nitrogen within the nucleated erythrocytes is from three to five times as great as the concentration in the corresponding plasma.

Evidence is quoted and further evidence given to show that the concentration of amino nitrogen is three to five times as great in the nucleated erythrocytes as in the corresponding serum.

It is assumed that the excess of amino-acid and other nitrogenous solutes of small molecular weight in the cells of nucleated types of blood over that in the plasma is largely confined to the nucleus.

It is suggested that the concentration of these substances as indicated in the nucleus is sufficient to maintain the osmotic tension of the same.

It is held that the conclusions reached as regards the maintenance of the osmotic tension of the nuclei of the avian erythrocytes are generally applicable to nuclei of tissue.

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EXPLANATION OF PLATES.

Figs. 1, 2, 9, 10, 12, 13, and 14 are photomicrographs. Figs. 3, 4, 5, 6, 7, 8, and 11 are drawings.

PLATE 1.

FIG. 1. Reaction in ventral ganglion of *Hirudo medicinalis* treated with 0.1 N silver nitrate in neutral solution.

FIG. 2. Reaction in nerve cell of sympathetic ganglion attached to cortex of suprarenal gland, treated with 0.1 N silver nitrate in neutral solution. Dog.

FIGS. 3, 4, AND 5. Reaction in ganglionic cells, treated with 0.1 N silver nitrate solution. Dog.

FIG. 6. Localization of chlorides in nerve cell and axones of Gasserian ganglion. Dog.

PLATE 2.

FIG. 7. Local zation of chlorides in the active gastric tubule. Dog.

FIG. 8. Localization of chlorides, phosphates, and carbonates in the active gastric tubule. Dog.

FIG. 9. Localization of chlorides, phosphates, and carbonates in the active proventriculus. Fowl.

FIG. 10. Localization of chlorides, phosphates, and carbonates in the resting proventriculus. Fowl.

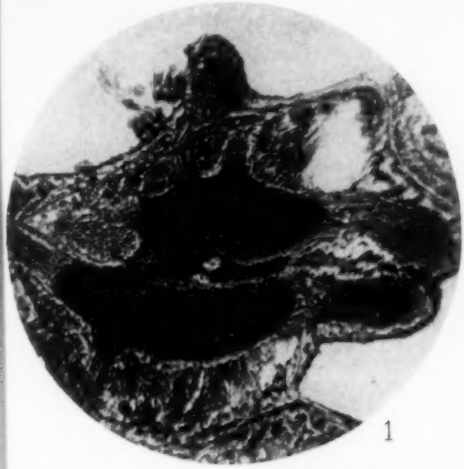
FIG. 11. Localization of chlorides, phosphates, and carbonates in the active proventriculus. Fowl.

PLATE 3.

FIG. 12. Localization of potassium about nerve cells of Gasserian ganglion. Dog.

FIG. 13. Two nuclei and one damaged nerve cell, photographed under ultramicroscope. *Hirudo medicinalis.*

FIG. 14. Two normal nerve cells, photographed under ultramicroscope. *Hirudo medicinalis.*



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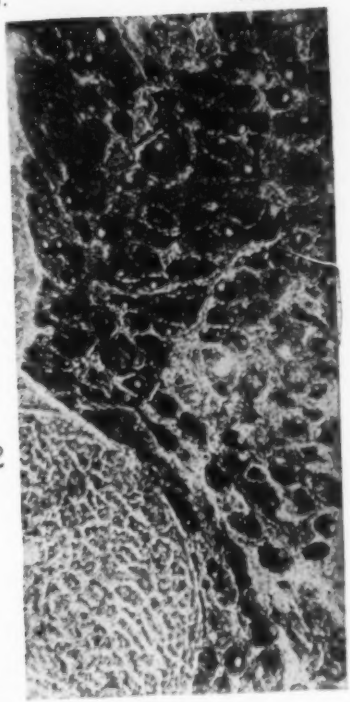

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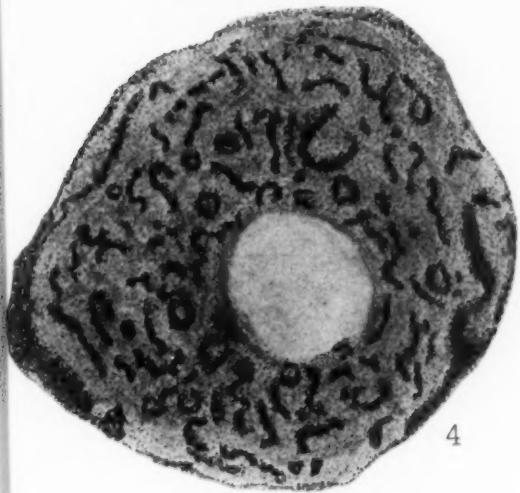

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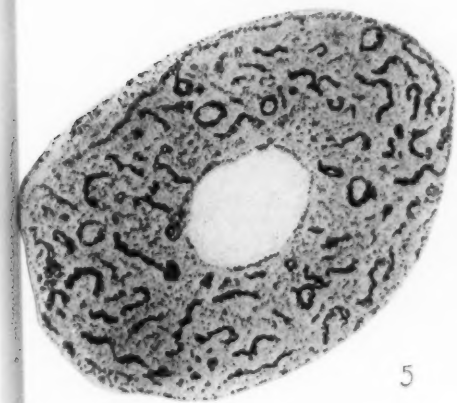

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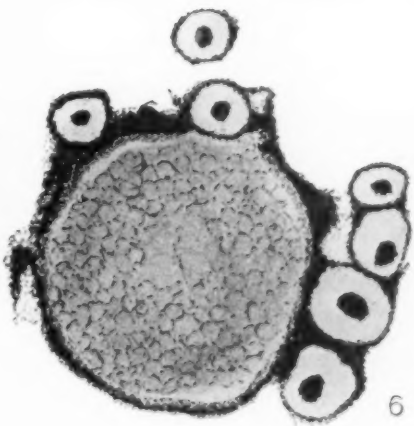

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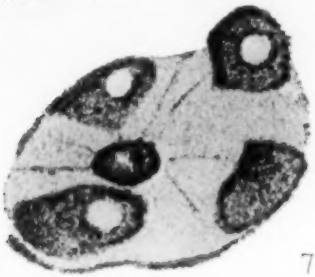

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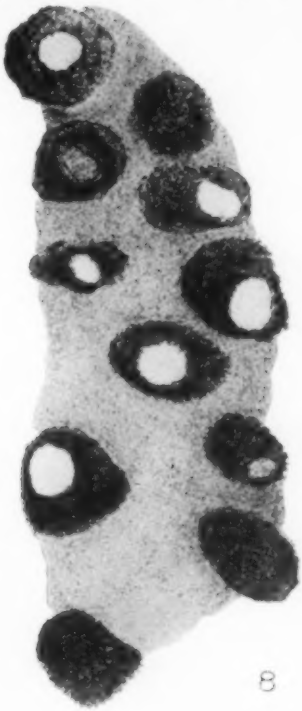

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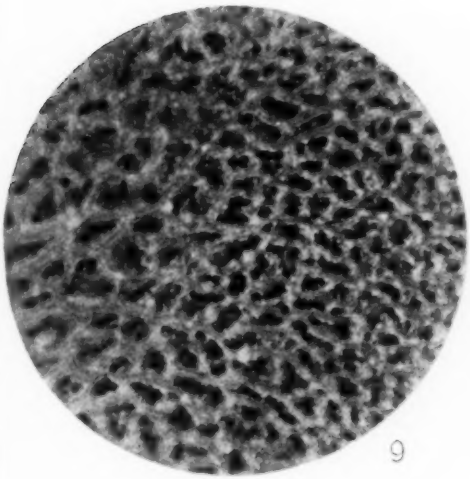

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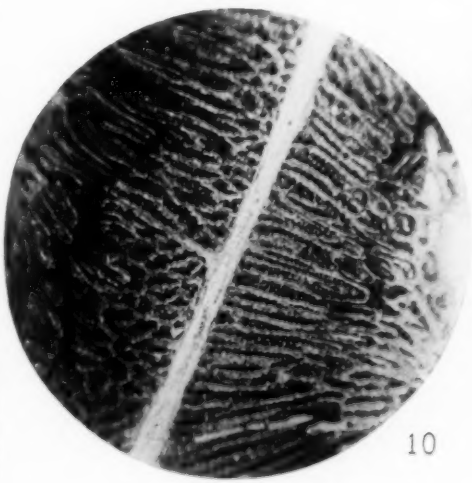

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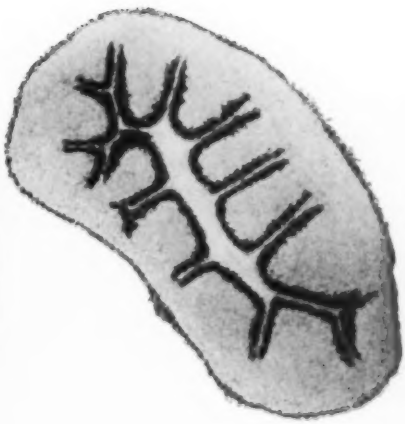

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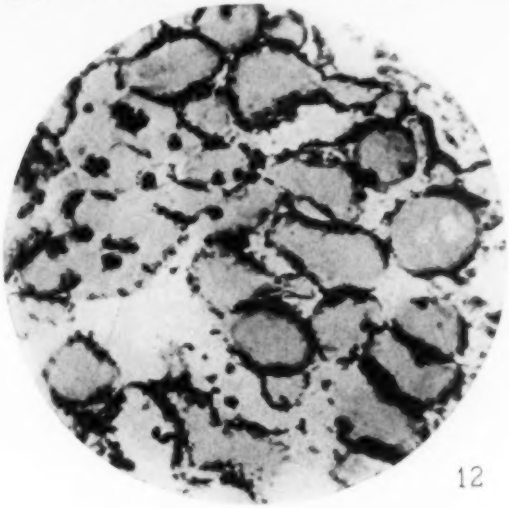

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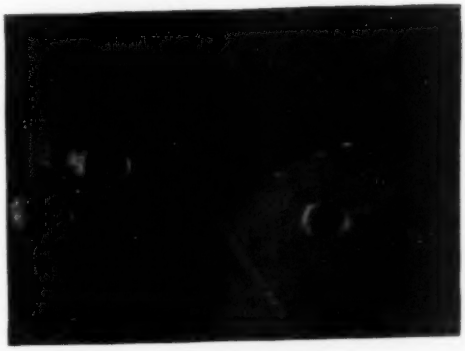

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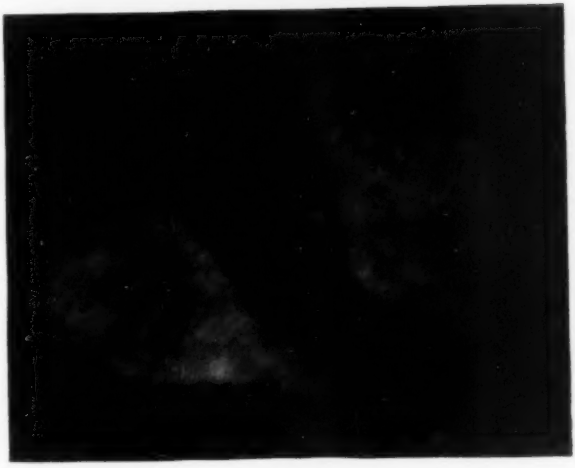

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